FUNCTIONAL GROUPS OF Na-K ATPase

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ABBREVIATIONS

ADP	Adenosine 5' diphosphate
AMPPNP	Adenylyl imido diphosphate
ATP	Adenosine 5' triphosphate
ATPase	Adenosine triphosphatase
Azo-bis	p-Azophenyl dimaleimide
Bis	Bis maleimidomethyl ether
CDTA	trans 1,2 Diaminocyclohexane N,N,N',N' tetraacetic acid
CuP	Cupric (o- phenanthroline)$_2$ sulfate
DTE	1,4 Dithio erythritol
DTNB	5,5' dithiobis (2-nitrobenzoic acid)
EDTA	1,2 ethylenediamine N,N,N',N' tetraacetic acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
K-NPPase	Potassium stimulated 4-nitrophenylphosphatase
Na-K ATPase	Sodium plus potassium stimulated adenosine triphosphatase
NEM	N-ethylmaleimide
NPP	4-Nitrophenylphosphate
NPPase	4-Nitrophenylphosphatase
P_i	Inorganic phosphate
SDS	Sodium dodecylsulfate
TCA	Trichloro acetic acid
Tris	Tris (hydroxymethyl) aminomethane

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CONTENTS

The enzyme system ($Na^+ + K^+$) activated ATPase plays a role in the transport of Na^+ and K^+ ions across the animal plasma membrane. It is a membrane bound enzyme. Since the discovery of its transport function, much effort has been expended to elucidate its mechanism of action.

From the enzymologist's point of view it is an interesting enzyme, since it converts chemical binding energy into motion. On the other hand it challenges protein chemists, since as an intrinsic membrane enzyme it is water-insoluble and requires lipids for activity, so that most of the usual protein purification techniques are unsuitable for its purification.

At the beginning of this study purified Na-K ATPase could only be obtained from sources not available to us in sufficient quantity (e.g. electric eel electroplax, dog kidney, dogfish rectal gland) Hence, the early work reported in this thesis is concerned with attempts to purify Na-K AtPase from sources more readily obtainable (cattle brain, rabbit kidney). The main part of the work, however, deals with the modification of the purified enzyme with group specific reagents.

The Na-K ATPase system shows, apart from the overall ATPase activity, stimulated by Na^+ plus K^+ ions and inhibited by ouabain, several other activities which may be explained as partial activities. Therefore, we have determined the effects of modification of certain functional groups on the overall enzyme activity as well as on the partial reactions. We shall also show that the study of the kinetics of the modification reactions can be useful for the elucidation of different conformational states of the enzyme. Finally, we shall deal with information obtained by treating the enzyme with bifunctional reagents.

In chapter 1 a more detailed introduction about various aspects of Na-K ATPase system is given. The methods used for the determination of the various activities and parameters of the enzyme will be described in chapter 2. In chapter 3 our attempts to purify the enzyme from cattle brain microsomes and the successful purification of the enzyme from rabbit kidney outer medulla microsomes will be described. The purified Na-K

ATPase will be characterized in chapter 4.

In subsequent chapters the results of modification of various functional groups are reported. Chapter 5 deals with the effects of treatment with N-ethylmaleimide on Na-K ATPase and related activities. Chapter 6 describes the results of the modification of sulfhydryl groups with 5,5'dithiobis(2-nitrobenzoic acid) (DTNB). Combined treatment with N-ethylmaleimide and with DTNB has resulted in a classification of (vital) sulfhydrylgroups (chapter 7). Modification of arginine residues with 2,3 butanedione is the subject of chapter 8. Subunit composition and mobility can be studied by reaction with bifunctional reagents. Effects of reaction with a bifunctional sulfhydryl reagent on the activity and the gel-electrophoretic protein pattern of the enzyme are reported in chapter 9.

In the final chapter (chapter 10) we shall discuss the implications of these findings for our understanding of the structure of the enzyme, its mechanism of action and the transport of Na^+ and K^+ ions across the plasma membrane.

CHAPTER I

GENERAL ASPECTS OF Na-K ACTIVATED ATPase

1.1 Introduction

1.1.1 Discovery and function

In virtually all animal cells a gradient of Na^+ ions exists across the cell membrane, the internal Na^+ concentration being much lower than the external one. On the other hand, the intracellular K^+ concentration is mostly higher than extracellularly. These gradients across the cell membrane are of physiological importance. The K^+ gradient determines in most cases the potential difference across the plasma membrane. In excitable tissues like nerve (Hodgkin, 1964) and the electric organ of the electric eel Electrophorus electricus (Schoffeniels, 1959) this potential difference is essential for excitation. The existence of a Na^+ gradient across the plasma membrane is essential for several Na^+-coupled transport processes, e.g. of sugars in the intestinal epithelium (Bihler and Crane, 1962) or of amino acids in red blood cells (Vidaver, 1964). Since there is a continuous movement of Na^+ and K^+ ions along their gradients across the plasma membrane, the maintenance of the gradients requires active transport in the opposite direction with expenditure of energy.

Already in 1957 Skou reported that in crab nerve microsomes a Mg^{2+} ATPase activity is present, which is increased in the presence of both Na^+ and K^+ ions. He suggested that the increase in ATPase activity might reflect the active transport of Na^+ and K^+ ions across the membrane. This suggestion was reinforced by his observation that the increased ATPase activity is inhibited by the cardiac glycoside ouabain (Skou, 1960), which had been shown to inhibit active cation transport in erythrocytes (Schatzmann, 1953). Post et al. (1960) and Dunham and Glynn (1961) showed the equivalence between the active fluxes of Na^+ and K^+ ions across erythrocyte membranes and the Na-K stimulated ATPase activity. In squid giant axon ouabain also inhibits Na^+ efflux (Caldwell and Keynes, 1959) due to blocking of the Na^+ pump (Martin and Shaw, 1966). Ouabain also

inhibits the Na^+ and K^+ stimulated ATPase activities in nerve homogenates (Skou, 1960).

It is now generally accepted that the Na-K ATPase system transports Na^+ and K^+ ions across cell membranes. The enzyme activity is inhibited by cardiac glycosides like ouabain and digitoxin, and also by erythrophleum alkaloids like erythrophleine and cassaine (Bonting et al., 1964a). The enzyme pumps 3 Na^+ ions per molecule ATP hydrolyzed (Bonting, 1970). In the case of erythrocytes the transport appears to be electrogenic, since only 2 K^+ ions are transported per 3 Na^+ ions (Sen and Post, 1964; Whittam & Ager,1965).This is also the case for Na-K ATPase contained in phospholipid vesicles (Goldin, 1977).

The enzyme system also plays a role in secretory processes. By comparison of Na-K ATPase activities in various tissues of the cat, Bonting et al. (1961) demonstrated that although the highest activities occurred in brain, there was also considerable activity in secretory organs. In secretory organs the Na-K ATPase activity may result in a net transcellular ion transport, so that Na^+ ions are retained (kidney) or secreted (salt gland, rectal gland). The active Na^+ transport can in certain cases be followed by a passive water flux. A positive correlation can be found between inhibition of fluid secretion and inhibition of Na-K ATPase activity by ouabain for aqueous humor (Simon et al., 1962, Bonting and Becker, 1964), cerebrospinal fluid (Vates et al., 1964) and pancreatic fluid (Ridderstap and Bonting, 1969). This proves that Na-K ATPase activity plays a role in fluid secretion in these cases.

In recent years some extensive reviews have been published on Na-K ATPase: the physiological role in cation transport (Bonting, 1970); the mechanism of action (Dahl and Hokin, 1974; Skou, 1975; Glynn and Karlish, 1975); the purification (Jørgensen, 1975a) and the pharmacological aspects (Schwartz et al., 1975).

1.1.2 Localization

Na-K ATPase activity has been demonstrated in various tissues from mammalians as well as from other classes of animals, like avians (Hokin, 1963, Bonting et al., 1964b), teleosts (Glynn, 1962; Epstein et al., 1967), elasmobranchii (Bonting, 1966), cephalopods (Bonting and Caravaggio, 1962) and arthropods (Skou, 1957; Liebermann, 1967). Since Na^+ and K^+ are trans-

ported across the plasma membrane, the transporting enzyme should be located in this membrane. Proof for the localization of Na-K ATPase in the plasma membranes is found in the enrichment of this enzyme activity in membrane fractions of cell homogenates, as in erythrocytes (Post et al., 1960), cardiac muscle (Portius and Repke, 1962), brain (Schwartz et al., 1962). Evidence for this has also been obtained from cytochemical techniques (Ernst, 1972a and b; Quinton et al., 1973; Quinton and Tormey, 1976; Kyte, 1976a and b). It does not occur in cell organelles like nucleus or mitochondria, and hence it is now considered a 'marker enzyme' for plasma membranes.

1.1.3 Purification

Na-K ATPase is a membrane bound enzyme, located in the plasma membrane. Tightly bound membrane proteins are notoriously difficult to purify. For application of the common protein purification techniques, the protein must either be removed from the surrounding membrane by solubilization with detergents, or else all other membrane proteins must be removed, leaving the enzyme in its membrane lipid environment.

Both techniques have been applied succesfully in the purification of Na-K ATPase. The first method, detergent solubilization has been applied to dog kidney outer medulla (Kyte, 1971; Lane et al., 1973), dogfish rectal gland (Hokin et al., 1973), electric eel electroplax (Dixon and Hokin, 1974; Perrone et al., 1975) and pig brain cortex (Nakao et al., 1973). The non-ionic detergent Lubrol has been applied to solubilize the Na-K ATPase in all cases, except in that of kidney outer medulla, where the anionic detergent deoxycholate was used. Drawback of this approach is that the resulting preparations are labile, unless the detergent is removed in such a way, that a lipid containing enzyme preparation is formed. The second method, detergent extraction of other proteins, has been applied to kidney outer medulla of sheep, rabbit and dog (Jørgensen, 1974a), guinea pig (Hayashi et al., 1977) and to nasal gland of duck (Anas platyrinchos) (Hopkins et al., 1976). It yields an active and stable enzyme preparation, which consists of membrane fragments with a full lipid complement.

1.2 Reaction mechanism

1.2.1 Models

For the Na-K ATPase reaction several models have been developed, some more directed to the transport of ions (Opit and Charnock, 1965; Jardetzky, 1966; Albers et al., 1968; Lowe, 1968; Stein et al., 1973; Skou, 1975) and others more directed to the hydrolysis of ATP (Post et al., 1969; Kanazawa et al., 1970; Robinson, 1971b; Repke and Schön, 1973; Lindenmayer et al., 1974; Albers et al., 1974; Fukushima and Tonomura 1975; Jørgensen, 1977). Some of the latter reaction models also include ion translocation steps.

Since the definitive model for the Na-K ATPase mechanism has not yet been developed, we shall limit ourselves to a model, in which the overall Na-K ATPase activity and several partial reactions are included (fig. 1.1). This model is a modified version of that suggested by Post et al. (1972). This reaction scheme includes the binding of ATP to the enzyme (step 1), followed by phosphorylation (step 2) and dephosphorylation of the enzyme (step 3). Two different conformations of the enzyme are assumed (E_1 and E_2). The conformational change $E_1 \rightarrow E_2$ could be associated with the translocation of Na^+ and K^+ ions. An addition to the scheme of Post et al. (1972) is the included K^+-stimulated 4-nitrophenylphosphatase activity (see section 1.4.3b). The main objection against this reaction scheme is that the $E_1 \sim P \rightarrow E_2 - P$ transition, which is supposed to be the transition of a 'high energy' phosphorylated intermediate to a 'low energy' phosphorylated intermediate, does not seem to occur. Kanazawa et al. (1970) and Fukushima and Tonomura (1975) suggest a different reaction scheme, in which this transition of high energy to low energy phosphate bond does not occur.

When discussing the Na-K ATPase reaction and its partial activities, one should always bear in mind that conclusions drawn from observations on membrane fragments, where no net ion transport can occur, need not necessarily apply to the situation in an intact cell membrane, where net transport does occur.

1.2.2 Properties

Na-K ATPase activity in membrane fragments can be measured as the hydrolysis of the terminal phosphate group of ATP, catalyzed by the enzyme in the presence of Na^+, K^+ and Mg^{2+} ions. This ATPase activity is

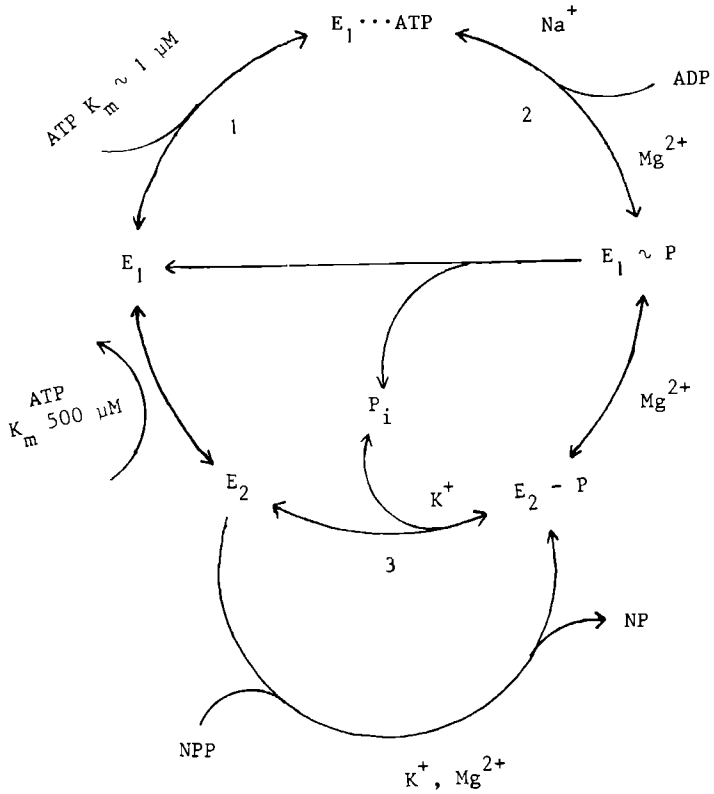


Fig. 1.1 Na-K ATPase reaction cycle. In this scheme, the K^+ stimulated 4-nitrophenylphosphatase reaction is included. E_1 and E_2 stand for different conformations of the enzyme. 1, 2 and 3 indicate steps as mentioned in text.

specificially inhibited by cardiac glycosides like ouabain and digitoxin. Maximal ATPase activity can be measured at a Mg : ATP ratio of 1 : 1 (Bonting, 1970, p. 262) and a Na : K ratio between 5 and 10 (Dahl and Hokin, 1974). In most assays the ATP concentration lies between 2 and 5 mM, whereas the Na^+ concentration lies between 60-120 mM. The optimal pH for Na-K ATPase activity varies between 7 and 8 (Bonting, 1970, p 267). The optimal temperature may be up to 45°C (Schoner et al., 1967; Portius and Repke, 1967). At 37°C turnover numbers (mol ATP hydrylyzed per mol Na-K ATPase per minute) between 5000 and 15,000 are found (Albers et al., 1968; Bader et al., 1968; Matsui and Schwartz, 1968; Ellory and Keynes, 1969;

Barnett, 1970; Hansen et al., 1971; Jørgensen and Skou, 1971; Siegel and Josephson, 1972).

There is a considerable preference for ATP as substrate. Other nucleoside triphosphates, such as dATP, CTP, ITP, GTP, can poorly substitute for ATP (Matsui and Schwartz, 1966; Schoner et al., 1968; Towle and Copenhaver, 1970).

The requirement for Na^+ is absolute, no other cation can substitute for it (Post et al., 1965). K^+ ions can be replaced by several other monovalent cations as Rb^+, Cs^+, NH_4^+, Li^+ and Tl^+ (Skou, 1965; Britten and Blank, 1968; Skulskii et al., 1973) with variable effectiveness.

The presence of Mg^{2+} ions is essential for ATPase activity. Mg^{2+} can only be replaced by Mn^{2+} or Co^{2+} ions, but this replacement results in lower enzyme activity. Ca^{2+} cannot replace Mg^{2+} for the overall activity (Tobin et al., 1973; Tobin et al., 1975), and is inhibiting at concentrations over 0.1 mM.

Determination of the Na-K ATPase activity at varying concentrations of ATP has revealed two different apparent K_m values for enzyme activity, suggesting the existence of two ATP binding centers (Kanazawa et al., 1970; Robinson, 1976). The low affinity ATP binding center is considered as an allosteric modifier site by these investigators, since the high affinity site is the phosphorylation site (see below). The overall ATPase reaction can be reversed in such a way that ATP is formed from ADP and P_i (Taniguchi and Post, 1975).

1.3 Binding of cations

1.3.1 Binding of Na^+ ions

The binding of ions to Na-K ATPase can be determined by direct measurements (isotope techniques, spectroscopic techniques) or by indirect measurements. In the latter case the corresponding K_{diss} values of ions to their binding sites can be calculated from the activating or inhibitory effects on enzyme activity. The main difference between both methods is that during the indirect measurement other ions are also present that may influence the results (K_{diss} values). Also the model in which the K_{diss} values are calculated might influence the results.

Direct measurement of Na^+ binding to Na-K ATPase results in various K_{diss} values for the enzyme-Na complex. Ostroy et al. (1974) have studied

binding of Na^+ ions to ox brain and dogfish rectal gland Na-K ATPase, and report a K_{diss} value for Na^+ binding on ox brain microsomes of 4.0 ± 0.4 mM. Kaniike et al. (1976) report the existence of 3 Na^+ binding sites per molecule Na-K ATPase with an apparent K_{diss} of 0.23 mM. This value is found by direct measurement of Na binding on sheep kidney outer medulla Na-K ATPase. The involvement of these sites in Na^+ transport is derived from the influence of ATP (Ostroy et al., 1974) or ouabain (Kaniike et al., 1976) on the K_{diss} value for Na^+ binding. Also some other sites have been reported by the latter group, but these might be due to the presence of phospholipids (K_{diss} 17-31 mM).

The results from indirect measurement of Na^+ binding sites are more complicated. Various types of Na^+ binding sites with various affinities have been calculated from the Na^+ effects on phosphorylation by ATP (Foster and Ahmed, 1976), on the Na-K ATPase reaction (Robinson, 1970b, 1977; Fukushima and Tonomura, 1975) and on phosphorylation by P_i (Taniguchi and Post, 1975). Not all Na^+ binding sites need to exist simultaneously on the enzyme; their presence depends on the step in the ATPase cycle.

Binding of Na^+ ions is influenced by ATP (Ostroy et al., 1974), ouabain (Kaniike et al., 1976), MgATP, K^+ or Mg^{2+} ions (Robinson, 1970b, 1977; Fukushima and Tonomura, 1975).

1.3.2 Binding of K^+ ions

Matsui et al. (1977) have studied by direct measurements the binding of ^{42}K to purified canine kidney outer medulla Na-K ATPase preparations. They report 2 K^+ binding sites per ouabain binding site, which are blocked by bound ouabain. The apparent K_{diss} values for K^+ binding to these sites amounts to 50 μM. The authors leave some doubt about possible cooperative effects of bound K^+ ions on subsequent binding of K^+ ions.

By indirect measurement various K^+ binding sites have been determined with different affinities (Robinson, 1975a; Fukushima and Tonomura, 1975). The affinity of K^+ for its binding site may also depend on the presence of bound Na^+ ions (Fukushima and Tonomura, 1975), or on the partial reaction of the ATPase cycle (Robinson, 1975).

Jørgensen (1975c) has shown that addition of either Na^+ or K^+ ions to pure Na-K ATPase preparations leads to different enzyme conformations.

1.3.3 Binding of Mg^{2+} ions

Magnesium ions bind to Na-K ATPase at a site with $K_{diss} \approx 1$ mM (Grisham and Mildvan, 1974; Robinson, 1974; Kuriki et al., 1976). There is one Mg^{2+} binding site per mol Na-K ATPase with this affinity. The affinity of Mg^{2+} for its binding site is influenced by Na^+ and K^+ ions. When precautions are taken to avoid the presence of these ions, the K_{diss} amounts to 150 ± 15 μM (Grisham and Mildvan, 1975). Binding of Mg^{2+} ions to the enzyme is associated with a large change in enthalpy (-49 kcal.mol^{-1}). This may indicate that a conformational change of the enzyme is induced by the binding of Mg^{2+} (Kuriki et al., 1976).

Additional Mg^{2+} binding sites with far lower affinity have been suggested (Robinson, 1974; Grisham and Mildvan, 1974, 1975). Some of these sites may represent phospholipids (Grisham and Mildvan, 1974, 1975) or are inhibitory sites (Robinson, 1974).

1.4 Partial steps of the Na-K ATPase reaction

1.4.1 Binding of ATP

Na-K ATPase, in the absence of Na^+, K^+ and Mg^{2+} shows a high affinity for ATP (K_{diss} 0.12-0.22 μM) (Hegyvary and Post, 1971; Nørby and Jensen, 1971; Jensen and Nørby, 1971). This affinity is almost unaffected by the addition of Na^+ ions (Hegyvary and Post, 1971; Nørby and Jensen, 1974) but is markedly lowered by the addition of K^+ ions (Nørby and Jensen, 1971). Na^+ and K^+ ions are competitive with each other with respect to their effects on the affinity for ATP (Hegyvary and Post, 1971). By comparing the affinity of this binding site for ATP with that for other nucleotides, Hegyvary and Post as well as Jensen and Nørby have found that the 6 amino group of the purine moiety and the β phosphate group are required for binding.

ADP also shows affinity for the enzyme (Hegyvary and Post, 1971; Kaniike et al., 1973). The affinity for ADP is increased by Na^+ ions and decreased by K^+, Mg^{2+} or Ca^{2+} ions. Na^+ and K^+ are competitive in their effects on ADP-binding. The effect of Mg^{2+} cannot be reversed by the addition of Na^+ ions (Kaniike et al., 1973).

The binding of ATP represents the first step of the Na-K ATPase reaction. Some investigators suggest a change of enzyme conformation induced by the binding of ATP (Skou, 1971, 1974; Kanazawa et al., 1970).

1.4.2 Na^+ dependent phosphorylation by ATP

The Na-K ATPase enzyme can be phosphorylated by ATP in the presence of Na^+ and Mg^{2+} ions (Albers et al., 1963; Hokin et al., 1965; Post et al., 1965; Nagano et al., 1967; Kanazawa et al., 1970). The optimal pH for phosphorylation is around 7.4. Since the concentration giving for maximal phosphorylation is low (about 100 μM ATP), the high affinity ATP binding site seems to be involved in the phosphorylation process. The receptor of the γ phosphate group from ATP seems to be a β aspartyl residue (Post and Kume, 1973; Nishigaki et al., 1974; Degani et al., 1974). The phosphorylated enzyme is stable at pH < 5 (e.g. after TCA denaturation). A heat stable phosphorylation product has also been described (Brodsky and Sohn, 1974). The phosphorylation of the enzyme by ATP seems to be the second step in the ATPase reaction cycle.

1.4.3 Dephosphorylation after phosphorylation by ATP

Three processes must be considered in connection with the dephosphorylation of the phosphorylated enzyme: ADP-ATP phosphate exchange, Na^+ stimulated ATPase activity and K^+ stimulated dephosphorylation. These three processes can be determined under different conditions. The phosphorylated enzyme can react with ADP (when present in excess) under formation of ATP and dephosphorylated enzyme, which amounts to an ADP-ATP phosphate exchange activity (Fahn et al., 1966a and b; Stahl, 1967, 1968; Banerjee and Wong, 1972). This activity requires Na^+ ions and lower concentrations (0.1-0.2 mM) of Mg^{2+} ions. The reaction amounts to a reversal of the phosphorylation by ATP, hence it would not normally be the third step in the ATPase reaction cycle.

There is also a Na^+-stimulated ATPase activity, which occurs in the absence of K^+ and is inhibited by ouabain. At low concentrations of ATP (< 100 μM) this activity is inhibited by K^+ ions (Neufeld and Levy, 1969; Post et al., 1972). The Na^+ ATPase activity does not seem to represent a separate step in the Na-K ATPase cycle, but rather to include the binding of ATP as well as the Na^+ dependent phosphorylation of the enzyme by ATP (step 1 and 2), followed by spontaneous dephosphorylation.

The third type of dephosphorylation reaction is enhanced by the addition of K^+ ions (Post et al., 1969; Klodos and Skou, 1975, 1977). Some investigators assume the involvement of a previous conformational

change of the phosphorylated enzyme under the influence of Mg^{2+} ions (Post et al., 1969). They present evidence that part of the phosphorylated enzyme would be in an ADP-sensitive form, and another part in a K^+-sensitive form, when the conformational change is blocked by complexing all free Mg^{2+} ions. Klodos and Skou (1975) deny the existence of two different conformations of the phosphorylated enzyme, since they find that in the presence as well as in the absence of Mg^{2+} ions both ADP and K^+ ions stimulate the complete dephosphorylation of the phosphorylated enzyme. Their results confirm the earlier results of Fukushima and Tonomura (1973). The K^+ enhanced dephosphorylation may well represent the third step in the reaction cycle (see fig. 1.1).

1.4.4 Phosphorylation by inorganic phosphate

The enzyme Na-K ATPase can be phosphorylated by P_i. This phosphorylation occurs at pH 7.0-8.0 in the presence of P_i and Mg^{2+} ions (Lindenmayer et al., 1968; Post et al., 1975). The phosphoenzyme formed by Na-K ATPase and P_i + Mg^{2+} is stabilized by the presence of ouabain (Lindenmayer et al., 1968). Presence of Na^+ or K^+ ions lowers the amount of enzyme phosphorylated by P_i and Mg^{2+} (Post et al., 1975). Maximal phosphorylation of the enzyme is reached with 1 mol phosphate bound per mol Na-K ATPase. After digestion of protein phosphorylated by P_i by pepsin, the same phosphorylated fragments were found as after digestion with pepsin of enzyme phosphorylated by ATP (Post et al., 1969; Chignell and Titus, 1969; Siegel et al., 1969).

The phosphorylation by P_i seems to represent the reversal of the last step of the ATPase activity, the dephosphorylation of the phosphorylated enzyme. The group to which P_i binds is not yet known, but P_i-phosphorylated enzyme is rather stable in acid (Post et al., 1969).

1.4.5 K^+-stimulated 4-nitrophenylphosphatase activity

In addition to ATP, several monophosphates can be hydrolyzed by the enzyme Na-K ATPase. Hydrolysis has been described from acetylphosphate (Izumi et al., 1966; Nagai et al., 1966; Sachs et al., 1967; Israel and Titus, 1967), carbamylphosphate (Yoshida et al., 1966; Izumi et al., 1966), 4-nitrophenylphosphate (Nagai et al., 1966; Fujita et al., 1966; Albers and Koval, 1966; Robinson, 1973), 2,4 dinitrophenylphosphate (Gache et al.,

1977), β-(2-furyl)acryloylphosphate (Gache et al., 1977), umbelliferone-phosphate (Pitts, 1974) and 3-O-methylfluoresceinephosphate (Huang and Askari, 1975). All these 'phosphatase' activities have in common that they are inhibited by ouabain. The cation requirement for optimal hydrolysis is not the same for all monophosphates, e.g. acetylphosphate is hydrolyzed in the presence of Na^+ ions plus Mg^{2+} ions, as well as in the presence of K^+ ions and Mg^{2+} ions, whereas 4-nitrophenylphosphate is only hydrolyzed in the presence of K^+ and Mg^{2+} ions (Formby and Clausen, 1968). The rate of hydrolysis relative to that of ATP varies. Some of these compounds (2,4-dinitrophenylphosphate and β-(2-furyl)acryloylphosphate) are hydrolyzed faster than ATP in the presence of Na^+, K^+ and Mg^{2+} ions (Gache et al., 1977), others at a lower rate.

We shall describe the K^+ stimulated 4-nitrophenylphosphatase activity more in detail, as this activity has been used by us as a parameter for the final step in the Na-K ATPase reaction. The 4-nitrophenylphosphatase activity requires Mg^{2+} and K^+ as cofactors. As with Na-K ATPase activity this phosphatase activity is inhibited by ouabain (Nagai et al., 1966; Fujita et al., 1966; Albers and Koval, 1966). The optimal pH is between pH 7 and 8 (Fujita et al., 1966; Sachs et al., 1967).

Addition of Na^+ ions to the phosphatase assay medium at optimal K^+ concentration inhibits phosphatase activity. At suboptimal K^+ concentrations the activity is enhanced by addition of minor amounts of Na^+ ions. Addition of low concentrations of ATP ($\leq$ 0.1 mM) in this case may increase the phosphatase activity (Nagai and Yoshida, 1966; Yoshida et al., 1969; Robinson, 1970a). K^+ can be replaced as the activating ion by Cs^+, Rb^+, Li^+ or Tl^+ ions with variable effectiveness (Koyal et al., 1971; Inturrisi, 1969), but Na^+ ions can hardly replace K^+.

It is possible to isolate a phosphoprotein originating from the reaction of 4-nitrophenylphosphate with the enzyme in the presence of ouabain (Inturrisi and Titus, 1970; Robinson, 1971a and b). Phosphorylation of the enzyme by 4-nitrophenylphosphate in the absence of ouabain has also been reported (Robinson, 1971a, see chapter 4).

Since the K^+ stimulated 4-nitrophenylphosphatase is stimulated by K^+ ions and hardly at all by Na^+ ions, it is assumed that it represents the final step in the ATPase reaction cycle.

1.5 Binding of ouabain

Already in 1953, Schatzmann reported the inhibition by cardiac glycosides of Na^+ and K^+ transport across the erythrocyte cell membrane. Later it became clear that the effect is exerted through inhibition of the Na-K ATPase activity (Post et al., 1960; Dunham and Glynn, 1961; Skou, 1960). In furhter studies ouabain has been used almost exclusively in view of its relatively high water solubility.

Ouabain binds to the outer side of the plasma membrane (Caldwell and Keynes, 1959; Hoffman, 1966). Prerequisite for ouabain binding is the presence of Mg^{2+} ions (Albers et al., 1968; Skou et al., 1971; Erdmann and Schoner 1973). Phosphorylation of the enzyme by either ATP or P_i increases the binding rate for ouabain (Albers et al., 1968; Schön et al., 1970). The enzyme-ouabain complexes formed under different phosphorylating conditions differ in properties (Akera and Brody, 1971; Akera et al., 1974; Schuurmans Stekhoven et al., 1976b). The ATP + Mg^{2+} + Na^+ dependent increase of ouabain binding is decreased upon addition of K^+ ions (Akera and Brody, 1971; Inagaki et al., 1974; Erdmann and Schoner, 1973) and also upon addition of ADP or non-phosphorylating ATP analogs (Hansen et al., 1971; Erdmann and Schoner, 1973; Tobin et al., 1973). The increase in affinity of the enzyme for ouabain by the presence of Mg^{2+} and P_i (Skou et al., 1971) is antagonized by Na^+ ions. The number of ouabain binding sites is equal to the number of ATP-phosphorylation sites (Kyte, 1972b). The dissociation constants as well as the dissociation rate constants of the enzyme-ouabain complexes may greatly differ for enzyme preparations from different organs and species (Tobin et al., 1972; Tobin and Brody, 1972; Schuurmans Stekhoven et al., 1976b). This may be connected with the different glycoside sensitivities of the enzyme in these organs and tissues. Binding of ouabain may cause a conformational change in the enzyme (Lindenmayer and Schwartz, 1970).

1.6 Relation to cation transport

1.6.1 Coupled Na^+-K^+ transport

The Na-K ATPase system transports Na^+ and K^+ ions across the plasma membrane, while utilizing ATP. Measurement of this transport requires a two compartment system. A commonly used system to study the transport

processes of the enzyme are erythrocytes, either intact cells or ghosts, and the squid giant axon, either intact or perfused. Four different types of transport have been distinguished and have been correlated with the overall and partial reactions of Na-K ATPase (Glynn and Karlish, 1975): coupled Na^+-K^+ transport, Na^+-Na^+ exchange, uncoupled Na^+ transport and K^+-K^+ exchange.

Coupled Na^+ efflux and K^+ influx is the normal form of the transport. From studies on erythrocyte ghosts it is clear that Na^+, Mg^{2+} and ATP need to be present on the inside and K^+ at the outside of the cell membrane, while ouabain inhibits the process on the outside of the membrane (Dunham and Glynn, 1961; Post et al., 1960).

The ratio of Na^+ ions to K^+ ions transported in erythrocytes is 3 : 2 (Sen and Post, 1964; Whittam and Ager, 1965). In squid giant axons a ratio varying between 1 : 1 and 5 : 1 can be found, depending on the concentrations of Na^+ and K^+ ions present at each side of the membrane (Sjodin and Beaugé, 1968; Brinley and Mullins, 1974). Reversal of the pump by external Na^+ coupled with synthesis of ATP from ADP + P_i is possible (Garrahan and Glynn, 1967a; Lew et al., 1970; Glynn and Lew, 1970; Land et al., 1970; Land and Whittam 1968).

1.6.2 Na^+-Na^+ exchange

This activity can be observed when the inner compartment of the system is loaded with Na^+, Mg^{2+}, ATP and ADP, and a high concentration of Na^+ ions present on the outside (Glynn, 1968; Brinley and Mullins, 1968; Baker et al., 1971). This activity consumes little or no energy (Garrahan and Glynn, 1967b) and coincides with an ADP-ATP phosphate exchange.

1.6.3 Uncoupled Na^+ transport

Under certain conditions (Viz. Na^+, Mg^{2+} and ATP inside, but no K^+ or Na^+ outside) a Na^+ efflux can be measured, which consumes ATP (Glynn and Karlish, 1976; Lew et al., 1973). This activity seems to be paralleled by the Na^+ stimulated ATPase activity mentioned earlier (section 1.4.2). Nevertheless some differences are observed: the K_m for ATP for the Na^+-stimulated ATPase activity is far lower; the Na^+ concentration required for maximal Na^+-stimulated ATPase activity is far higher and exceeds the inhibitory extracellular Na^+ concentrations (Karlish and Glynn, 1974;

Glynn and Karlish, 1976).

1.6.4 K^+-K^+ exchange

This activity can be demonstrated when K^+, ATP and P_i are present in the inner compartment, and K^+ ions are present externally. It does not consume energy (Glynn et al., 1970, 1971; Simons, 1974). Perhaps this activity coincides with the phosphorylation by P_i, which may occur under these conditions (Dahms and Boyer, 1973).

1.7 Structure of the enzyme complex

From radiation inactivation studies it is known that the Na-K ATPase system has a molecular weight of 250,000 (Kepner and Macey, 1968). SDS gel electrophoresis and gel filtration of SDS solubilized enzyme show that the enzyme molecule consists of two major polypeptides, a 90,000-100,000 protein, and a 40,000-60,000 glycoprotein (Jørgensen, 1974a; Perrone et al., 1975; Hayashi et al., 1977).

Some investigators (Hokin et al., 1973; Hopkins et al., 1976) report in addition a MW 10,000 component after gel filtration of SDS solubilized, highly pure enzyme. Also in SDS polyacrylamide gel electrophoresis according to the Laemmli method (1970) a substance in found by Hokin et al. (1973) and Dixon and Hokin (1974). It stains with Coomassie blue R250, has an absorbance at 280 nm, but does not react with Folin reagent. Its nature is unknown.

The two large polypeptides seem to be an integral part of the enzyme. This has been demonstrated in the first place by antibody studies. Antibodies raised against either one of the two polypeptides inhibit the Na-K ATPase activity (Rhee and Hokin, 1975; Kyte, 1974; Jean et al., 1975; Jean and Albers, 1977). The 100,000 polypeptide can be phosphorylated by ATP (Collins and Albers, 1972; Avruch and Fairbanks, 1972; Alexander and Rodnight, 1974; Tobin et al., 1975; Hopkins et al., 1976) and by P_i (Schuurmans Stekhoven et al., 1976a). It can also be labeled with a photoaffinity analog of ATP (Haley and Hoffman, 1974), with a photoaffinity analog of coumarin (Ruoho and Kyte, 1974), which is an inhibitor of Na-K ATPase activity, and with ouabain (Hegyvary, 1975, Kott et al., 1975). The 50,000 glycoprotein is not phosphorylated. Hence, the 100,000 polypeptide is often called the catalytic subunit. A Na^+ ionophore has been

isolated after tryptic digestion of the 50,000 glycoprotein subunit (Shamoo and Albers, 1973), suggesting that this subunit may play a specific role in Na^+ transmembrane transport.

Crosslinking experiments suggest that two 100,000 polypeptides are located next to each other (Giotta, 1976; Kyte, 1975). Since a crosslink has also been made between a 100,000 polypeptide and a 50,000 polypeptide (Kyte, 1972a), it appears that the enzyme system consists of two 100,000 polypeptides and at least one 50,000 polypeptide. Neither of the isolated subunits shows catalytic activity (for one exception: Nakao et al., 1973), suggesting that the full array of subunits is required for this activity. Kyte (1975) concludes from the fact that ouabain binds to the outer side of the membrane, and that antibody simultaneously binds at the inner side that the Na-K ATPase complex spans the membrane.

1.8 Lipid involvement in enzyme activity

The Na-K ATPase activity requires the presence of lipids for its activity. All highly purified Na-K ATPase preparations described so far contain a considerable amount of phospholipids. Electron microscopic pictures of these preparations show membrane structures (Hokin et al., 1973; Maunsbach and Jørgensen, 1974; Van Winkle et al., 1976; Hayashi et al., 1977).

The structure of Na-K ATPase is influenced by its phospholipid surrounding (Simpkins and Hokin, 1973). The Na-K ATPase activity requires a phospholipid surrounding (Wheeler, 1975; Wheeler and Walker, 1975; Wheeler et al., 1975; Ottolenghi, 1975). The phospholipid requirement of the partial activities differs from the requirement for the overall activity. Phosphorylation by ATP and K^+ stimulated phosphatase activity are less inhibited by delipidation than the overall Na-K ATPase activity (Wheeler, 1975; Wheeler and Walker, 1975; Jensen and Ottolenghi, 1976). The phospholipids adjacent to Na-K ATPase seem to be more fluid than those of the rest of the membrane (Grisham and Barnett, 1972).

The fatty acid residues of the phospholipids may influence the Na-K ATPase activity (Walker and Wheeler, 1975a). In Na-K ATPase preparations a temperature dependent transition of the activation energy for the ATPase reaction has been found, that is attributed to a change in fluidity of the fatty acid chains of the phospholipids (Gruener and Avi-Dor, 1966). This

temperature dependent transition has not been found for the K^+-stimulated 4-nitrophenylphosphatase activity (Barnett and Palazotto, 1974; Walker and Wheeler, 1975b).

Originally, it was believed that phosphatidylserine or at least a negatively charged phospholipid is essential, since reactivation of Na-K ATPase, by complete delipidation, occurs preferentially with phosphatidylserine or other negatively charged phospholipid (Taniguchi and Tonomura, 1971, Hokin and Hexum, 1972; Kimelberg and Papahadjopoulos, 1972, 1974; Roelofsen and van Deenen, 1973; Palatine et al., 1977). However, de Pont et al. (1973) has shown by selective enzymatic conversion of phosphatidyl serine (a negatively charged phospholipid) to phosphatidyl ethanolamine (a neutral phospholipid), that phosphatidyl serine is not essential for Na-K ATPase activity. Subsequently, they have shown in a similar way that the other negatively charged phospholipid phosphatidyl inositol is not essential either, but that a minimum of about 90 molecules phospholipid per enzyme molecule are required for maintenance of activity (de Pont et al., 1978). In agreement with this, activity is also retained after cholate mediated exchange of all phospholipids by phosphatidylcholine (Racker and Fisher, 1975; Hilden and Hokin, 1976; Goldin, 1977). In conclusion, we may say that a certain amount of phospholipids is essential for Na-K ATPase activity, but that the type is not critical.

1.9 Purpose of this study

The purpose of this study is twofold; In the first place: to establish a reliable purification method for Na-K ATPase, which yield a stable, highly pure preparation suitable for further studies of the reaction mechanism and related characteristics of the enzyme. At the start of this thesis project (1973) only a few purification procedures for Na-K ATPase had been described. The first part of the thesis (chapter 3 and 4) deals with this part of the project.

In the second place: chemical modification of purified Na-K ATPase to supply more information about the structure of the catalytic center of the enzyme and about its conformations. The results of the modification experiments are given in the second part of this thesis. The effects of treatment of purified Na-K ATPase with N-ethylmaleimide are reported in chapter 5. The effects of treatment with 5,5' dithiobis (2-nitrobenzoic

acid) are communicated in chapter 6, combined effects of treatment with N-ethylmaleimide with treatment with 5,5' dithiobis (2-nitrobenzoic acid) are described in chapter 7. The effects of treatment with 2,3 butanedione on Na-K ATPase activity and partial activities are subject of chapter 8.

More information about subunit interaction has been sought by the use of crosslinking reagents (bis maleimidomethyl ether, p-azophenyl N,N' di-maleimide and copper phenanthroline catalyzed disulfide formation). The results of these experiments are related in chapter 9. The last chapter (10) is a summary and a general discussion of the implications of our findings for the structure and mechanism of the Na-K ATPase system in relation to its transport function.

CHAPTER 2

DETERMINATION OF VARIOUS ACTIVITIES AND OTHER PARAMETERS OF THE Na-K ATPase ENZYME SYSTEM

2.1 Introduction

The enzyme Na-K ATPase displays, in addition to its overall enzymatic activity, some partial enzymatic activities and other parameters, which can be determined in order to obtain information about its mechanism of action. In this chapter the determinations of these parameters, as used in this thesis, will be described. Where necessary, the optimal assay conditions have been determined. Data on the limitations of the various assays are also included.

2.2 Materials

Tris-ATP and sodium-dodecylsulfate are obtained from Sigma (St. Louis, Mo., USA), (γ^{32}P)ATP (initial specific activity 3.0 Ci.mmol^{-1}) and (^{32}P)4-nitrophenylphosphate (initial specific radioactivity 10 Ci.mol^{-1}) are delivered by Radiochemical Center (Amersham, England). Carrier free $^{32}P_i$ in aqueous solution and Aquasol are purchased from NEN Chemicals (Frankfurt a.M., W-Germany). Selectron AE95 filters (1.2 µm pore seize) are purchased from Schleicher and Schüll (Dassel, W-Germany), Coomassie brilliant blue 250 is from Serva (Heidelberg, W-Germany). N,N,N',N',tetramethylethylenediamine is delivered by Fluka A.G. (Buchs SG, Switzerland). All other chemicals are from Merck (Darmstadt, W-Germany) and are of analytical grade.

Imidazole phosphate is prepared by neutralization of o-phosphoric acid with imidazole. 4-Nitrophenylphosphate (Na_2 salt) is converted to its imidazole salt by passage over a Dowex 50 column (H^+ form) and subsequent neutralizationof the free acid with imidazole. Radioactive counting is performed in a Philips 12-channel Liquid Scintillation Analyzer. Spectrophotometric determinations are performed on a Zeiss PMQ II spectrophotometer in 500 µl cuvets (1 cm lightpath).

2.3 Determination of Na-K ATPase activity

Na^+ plus K^+ stimulated, ouabain sensitive Mg^{2+} ATPase activity is determined as the difference in ATPase activity in two media, one yielding total ATPase activity and one yielding the ouabain insensitive rest ATPase activity (see. Bonting, 1970, p. 262, media A and E). The composition of both media is given in table 2.1

Table 2.1

COMPOSITION OF Na-K ATPase ASSAY MEDIA

medium A (total ATPase activity)	medium E (rest ATPase activity)
100 mM NaCl	100 mM NaCl
10 mM KCl	0.1 mM Ouabain
5 mM $MgCl_2$	5 mM $MgCl_2$
5 mM Na_2ATP	5 mM Na_2ATP
50 mM imidazole-HCl (pH 7.4)	50 mM imidazole-HCl (pH 7.4)

The Na-K ATPase activity is calculated as the difference between the activities in media A and E.

Two different assay methods have been used: methods I and II.

Method I.

The reaction is started by addition of the enzyme (up to 50 µl volume) to 400 µl of the assay medium, preincubated at 37°C. After a predetermined period of incubation at 37°C the ATPase reaction is stopped by addition of 1.5 ml cold 8.6% (w/v) solution of trichloro acetic acid (TCA). To each tube 1.5 ml of a freshly prepared solution of 9.6% (w/v) $FeSO_4.6H_2O$, 1.15% (w/v) ammonium heptamolybdate in 0.66 M H_2SO_4 is added, and after 30 min at room temperature the absorbance at 700 nm is determined. The amount of ATP hydrolyzed can be calculated from the 700 nm absorbance of standard inorganic phosphate solutions (0.625 and 1.25 mM P_i), treated in the same way. To correct for non-enzymatic phosphate production, endogenous phosphate and reagent stain, blanks are prepared by incubating 400 µl medium E and adding the enzyme after the addition of the TCA solution. The assay is adapted from the procedure described by Bonting (1970, p. 262-263). There is a linear relationship between the amount of P_i formed and the incubation

time at 37°C up to 25% hydrolysis of the initial 5 mM ATP (fig. 2.1).

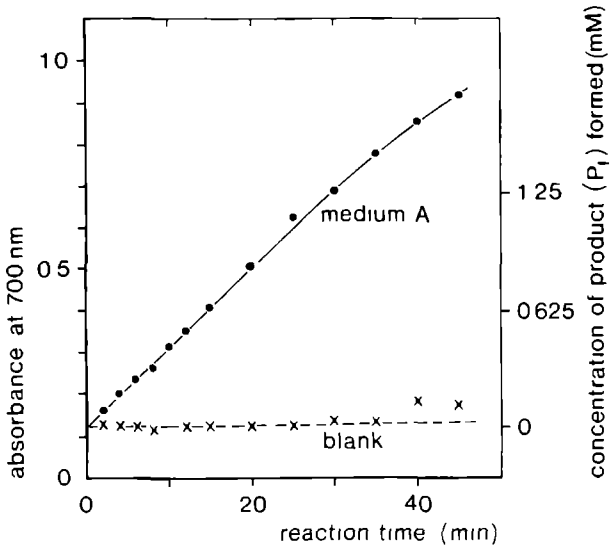


Fig. 2.1 Relationship between ATP hydrolysis and time of incubation at 37°C. Total ATPase activity for NaI treated cattle brain microsomes and blank values are shown. In NaI treated cattle brain microsomes the rest ATPase activity (determined in medium F) is very low. In this experiment it does not differ from the blank.

Method II

A radioactive assay method has been used in cases where a parallel determination of the Na^+ ATPase activity (section 2.4) is desirable. The procedure described as Method I is used, except that in addition to 5 mM ATP 1 µM (γ^{32}P)ATP is added to the assay media. After stopping the reaction and staining for phosphate, the reduced phosphomolybdate complex is extracted into 3 ml isobutanol (Neufeld and Levy, 1969). One ml aliquots of the isobutanol layer are added to 10 ml scintillation fluid (Aquasol), and the mixture is counted for ^{32}P in a liquid scintillation analyzer.

2.4 Na^+ stimulated ATPase activity

Na-ATPase activity is determined as the difference in P_i production at 37°C in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, 1 µM (γ^{32}P)ATP, 0.1 mM EDTA, 30 mM imidazole-HCl (pH 7.4) and a medium of the same

composition, containing in addition 0.1 mM ouabain. The P_i production is measured by stopping the reaction at the desired time by addition of 1.5 ml 8.6% (w/v) TCA in 0.1 mM H_3PO_4 to 400 µl reaction mixture. The $^{32}P_i$ formed is extracted into 3 ml isobutanol as described in the preceding section (method II). In some experiments, in which the inhibition of Na-ATPase activity by K^+ ions is determined, 10 mM KCl is added to the medium without ouabain. This assay is derived from the method described by Neufeld and Levy (1969). The amount of product formed never exceeds 5% of the original amount of ATP.

2.5 Phosphorylation by ATP

Phosphorylation of Na-K ATPase may be performed at 37°C (Skou and Hilberg, 1969; Blostein, 1968) or at 0°C (Nagano et al., 1967; Blostein, 1968; Fahn et al., 1968). All assays have in common that Na^+ and Mg^{2+} ions are required for phosphorylation of Na-K ATPase by (γ^{32}P)ATP. For phosphorylation at 37°C the phosphorylation medium with (γ^{32}P)ATP is mixed with 25 µl enzyme solution (both preincubated at 37°C). The final composition of the phosphorylation medium is 100 mM NaCl, 5 mM $MgCl_2$, 17 µM (γ^{32}P)ATP, 25 µg protein and 30 mM imidazole-HCl (pH 7.4). After incubation for 3 sec at 37°C phosphorylation is stopped by addition of 2 ml 5% (w/v) TCA. The mixtures are quantitatively transferred to a Selectron AE 95 filter by means of two additional volumes of 5% (w/v) TCA. The filters are washed four times with 5 ml 5% (w/v) TCA and are than dissolved in 10 ml scintillation fluid (Aquasol). Blanks are prepared by adding TCA to the reaction mixture before addition of (γ^{32}P)ATP. In fig. 2.2a the amount of phosphorylated protein as a function of the concentration of (γ^{32}P)ATP and the time of incubation is shown. Addition of 10 mM KCl to the phosphorylation medium decreases the amount of phosphorylated enzyme to zero. Since a steady state phosphorylation level is reached at approx. 17 µM ATP, this concentration is used for phosphorylation experiments. This concentration of (γ^{32}P)ATP offers the best compromise between achieving a high level of incorporation of radioactivity and keeping a low blank value.

Phosphorylation of the enzyme at 0°C is carried out in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, 20 µM (γ^{32}P)ATP, 5-25 µg protein and 30 mM imidazole-HCl (pH 7.4) in a final volume of 100 µl . Phosphorylation

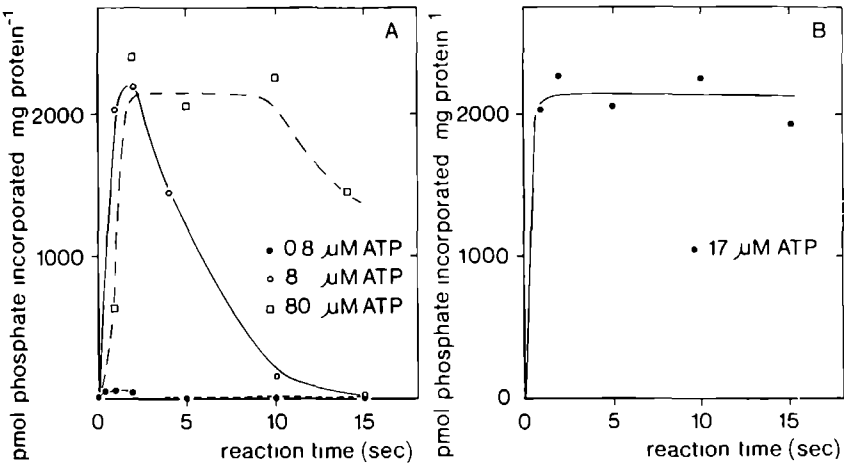


Fig. 2.2 Phosphorylation of Na-K ATPase by ATP.

a. Phosphorylation at 37°C. Purified rabbit kidney outer medulla Na-K ATPase is phosphorylated by ^{32}P ATP at three concentrations of ATP (--●--, 0.8 µM, —○—, 8 µM, - □ -, 80 µM ATP). Phosphorylation is plotted as a function of the incubation time.

b. Phosphorylation at 0°C. Purified rabbit kidney outer medulla Na-K ATPase is phosphorylated by ^{32}P ATP (17 µM). Phosphorylation is plotted as a function of the incubation time.

is started by adding the enzyme to the phosphorylation medium, both cooled to 0°C. After 15 sec the reaction is stopped by addition of 2 ml of 5% (w/v) TCA in 0.1 M H_3PO_4. The denatured protein is collected by filtration over a Selectron AE 95 (1.2 µm) filter. After washing the filter 3 times with 5 ml stopping solution, the filters are dissolved in scintillation fluid (Aquasol) and counted for ^{32}P in a liquid scintillation analyzer. Blanks are prepared by adding the stopping fluid before the protein to the medium. The maximal amount of protein on a filter never exceeds 25 µg. In fig. 2.2b the amount of phosphorylated enzyme is shown as a function of the reaction time. Addition of 10 mM KCl to the phosphorylation medium decreases the amount of enzyme phosphorylated, but never to zero. This may be due, either to a higher temperature sensitivity of the dephosphorylation than the phosphorylation reaction, or to a lowering of the

affinity of the enzyme for ATP in the presence of KCl (Nørby and Jensen, 1971) Since no other proteins than Na-K ATPase are present in pure preparations, the phosphorylation value upon addition of trichloroacetic acid prior the enzyme is chosen as the blank. The advantage of the low temperature method is that the phosphorylation level is maintained for a longer period than in the high temperature method (compare fig. 2.2a with fig. 2 2b)

2.6 Phosphorylation by P_i

Carrier free ^{32}P phosphoric acid in H_2O contains a minimal amount of radio-isotope contamination which adheres very strongly to protein This is apparent from the fact that excess $^{31}P_i$ cannot completely chase the radioactivity from the enzyme protein, which has previously also been observed by Skou and Hilberg (1969). This contamination is removed by mixing carrier free $^{32}P_i$ from stock with boiled rabbit kidney microsomes (final concentration 0.5 mg protein.ml^{-1}), followed by centrifugation. The supernatant contains little or no radioisotope contamination and is used as purified carrier free $^{32}P_i$.

Phosphorylation of purified Na-K ATPase by P_i is carried out by one of two slightly different techniques. a centrifugation and a filtration technique In the former method a medium of the following composition is used: 5 mM $MgCl_2$, 6.10^5cpm purified carrier free $^{32}P_i$, 50 µM imidazole phosphate, 300 µg protein.ml^{-1}, 50 mM imidazole-HCl (pH 7.0) in a final volume 500 µl. After 4 min at 0^oC the reaction is terminated by adding 100 µl 50% (w/v) TCA and 100 µl 0.1 M H_3PO_4, adjusted to pH 3.0 with NaOH. The precipitated enzyme is collected by centrifugation at 18,000 rpm ($40,000g_{max}$) for 15 min. The supernatant is removed and the pellet is washed twice by centrifugation with 700 µl 5% (w/v) TCA in 0.1 M H_3PO_4. The washed pellet is dissolved by heating it for 15 min at 90^oC in 1 ml 0.5 N NaOH. A 750 µl aliquot of this solution is acidified with 0.5 ml 1 N HCl and analyzed for ^{32}P after addition of 10 ml scintillation fluid (Aquasol). In 40 µl aliquots the protein content is determined according to the method of Lowry (microprocedure). Fig. 2.3 shows phosphorylation as a function of time at two P_i concentrations. For P_i concentrations between 1 and 100 µM a 4 min phosphorylation period is sufficient to reach equilibrium, which is in accordance with the results of Hegyvary (1976).

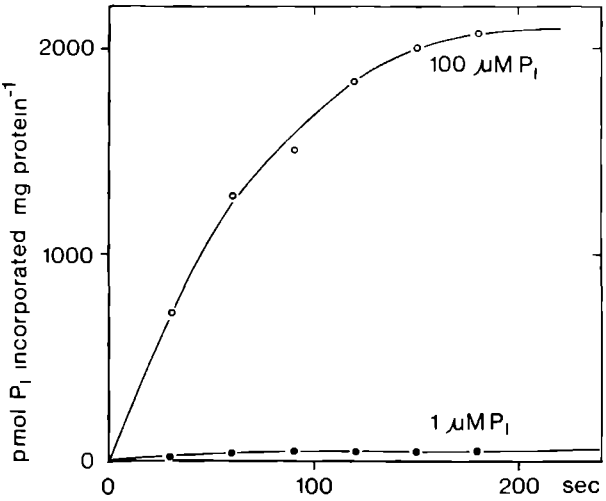


Fig. 2.3 Phosphorylation of Na-K ATPase by P_i. Purified rabbit kidney outer medulla Na-K ATPase is phosphorylated by P_i (-●-, 1 µM; -o-, 100 µM) at 0°C. Phosphorylation is plotted as a function of the incubation time.

A faster method, which requires less protein, makes use of filtration. Phosphorylation is performed in a medium containing: 4 mM $MgCl_2$, 50 µM imidazole phosphate, 6 x 10^5 cpm purified carrier free $^{32}P_i$, 25 µg protein (purified enzyme), 50 mM imidazole-HCl (pH 7.0) in a final volume 100 µl. Equilibrium is reached within 4 min at 0°C, at which time the reaction is stopped by adding 2 ml 5% (w/v) TCA in 0.1 M H_3PO_4. The phosphorylated protein is collected by filtration as described in section 2.5. Blanks are prepared by adding TCA solution before enzyme protein to the radioactive solution. The assay conditions are adapted from the method of Schuurmans Stekhoven et al. (1976a).

2.7 K^+ stimulated 4-nitrophenylphosphatase activity

This activity, which appears to represent the dephosphorylation reaction, is determined as the difference in 4-nitrophenol production at 37°C in a medium containing: 6 mM $MgCl_2$, 10 mM KCl, 5 mM 4-nitrophenylphosphate (imidazole salt), 1 mM CDTA and 30 mM imidazole-HCl (pH 7.4) and

in a medium of the same composition but without KCl and with 0.1 mM ouabain added. The 4-nitrophenol concentration is determined by measuring the 410 nm absorbance after stopping the reaction by addition of 2 ml 0.5 N NaOH to 400 µl reaction mixture. Solutions with known concentration of 4-nitrophenol (0.125 mM and 0.25 mM) are used as standards.

This assay is derived from the method described by Nagai et al. (1966), but conditions have been made optimal for use with the purified Na-K ATPase preparation as described in chapter 4. The amount of 4-nitrophenylphosphate hydrolyzed during the assay never exceeds 5% of the original amount present.

2.8 Phosphorylation by 4-nitrophenylphosphate

Phosphorylation by (^{32}P)4-nitrophenylphosphate is carried out in a medium containing: 2 mM (^{32}P)4-nitrophenylphosphate, 340 µg protein, 25 mM imidazole-HCl (pH 7.4) and other additives as indicated, in a final folume of 500 µl. Phosphorylation is allowed to proceed at either 37^{o} or 0^{o}C for a given length of time, usually 1 min. The reaction is stopped by adding 5 ml of a solution containing 5% (w/v) TCA and 10 mM unlabeled 4-nitrophenylphosphate. The precipitated protein is sedimented by centrifugation for 15 min at 48,000g$_{max}$. The pellet is washed twice, first with 3 ml, then with 1 ml of the stopping solution, each time followed by 15 min centrifugation at 48,000g$_{max}$. The washed pellet is dissolved and the amount of phosphorylated protein is determined as described in section 2.6. This determination is adapted from the procedures described by Inturrisi and Titus (1970) and by Robinson (1971a), and is discussed in chapter 4.

2.9 Protein determination

2.9.1 Protein concentration

Protein concentrations are determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard. In the absence of interfering substances the protein samples are diluted with water to 400 µl. When interfering substances (e.g. sucrose, glycerol) are present, they are removed by precipitating the protein with 5% (w/v, final concentration) TCA as described by Jørgensen (1975b). The protein precipitate is sedimented by centrifugation at 3,000g for 30 min in an IEC

clinical centrifuge equipped with swinging bucket rotor. After washing twice with 5% (w/v) trichloroacetic acid, the precipitate is dissolved in 400 µl 0.1 N NaOH (15 min at 90°C). This procedure is also applied in order to concentrate protein in samples with concentrations below the detection level.

To the 400 µl protein solution 2 ml solution containing 0.01% (w/v) $CuSO_4.H_2O$, 0.02% (w/v) Na-K tartrate and 2% (w/v) Na_2CO_3 in 0.1 M NaOH is added. After 10 min at room temperature, 200 µl of 1 N Folin-Ciocalteu solution is added and the absorbance at 500 nm or 750 nm is measured.

When only very small amounts of protein are available a microprocedure is used, in which all volumes are divided by ten.

2.9.2 Protein composition

Protein composition of the samples is determined by polyacrylamide gel electrophoresis (PAGE) after solubilization in sodium dodecylsulfate (SDS). Two methods have been used, the method of Laemmli (1970) and the method of Davies and Stark (1970). In the former method electrophoresis is performed in glass cylinders (10 cm x 0.7 cm Ø) or in slabs (1 mm x 16 cm x 16 cm). The separating gel contains: 8.75% (w/v) acrylamide, 0.234% (w/v) N,N'-methylene bisacrylamide, 0.025% (v/v) N,N,N',N',tetramethylethylenediamine, 0.375 M Tris-HCl (pH 8.8), 0.1% sodium dodecylsulfate and 0.03% (w/v) ammonium peroxodisulfate. The stacking gel contains: 3% acrylamide, 0.08% (w/v) N,N' methylene bisacrylamide, 0.1% (v/v) N,N,N',N',tetramethyl-ethylenediamine, 0.12 M Tris-HCl (pH 6.8) and 0.03% (w/v) ammonium peroxo-disulfate. The electrode buffer contains: 0.19 M glycine (adjusted with Tris to pH 8.3) and 0.1% (w/v) sodium dodecylsulfate. Protein is solubilized at 37°C in 2-16 hrs in a medium containing 5% (v/v) glycerol, 1.5% sodium dodecylsulfate, 0.0312 M Tris-HCl (pH 6.5), 5% (v/v) ß mercaptoethanol. Before application to the gel a small amount of tracking dye (0.1% (w/v) Bromophenol blue in ethanol) is added to the protein solution. The separating gels are polymerized overnight at room temperature under an isobutanol layer. The stacking gel is polymerized for 0.5 hr at room temperature under isobutanol. The volume of the stacking gel is at least twice that of the sample to be applied.

After application of the sample the current is set at 1 mA per 40 mm^2 gel surface. When the tracking dye enters the separation gel the current

is doubled. When the tracking dye reaches the bottom of the gel, the gels are removed, and the position of the tracking dye is marked with India ink. The following proteins are used to calibrate the gel: phosphorylase a (MW 94,000), bovine serum albumin (MW 68,000), pyruvate kinase (MW 57,000) and ovalbumin (MW 43,000).

Gel electrophoresis according to Davies and Stark (1970) is performed in slabs (1 mm x 16 cm x 16 cm). The gel contains. 3 5% (w/v) acrylamide, 0 135% (w/v) N,N'methylene bisacrylamide, 0 033% N,N,N',N',tetramethylethylenediamine, 0.1 M sodium tetraborate, 0.1 M sodium acetate (pH 8.5) and 0.075% (w/v) ammonium peroxodisulfate. The electrode buffer contains. 0.1 M sodium tetraborate, 0.1 M sodium acetate and 0.1% (w/v) sodium dodecylsulfate, adjusted to pH 8.5 with acetic acid. The protein is dissolved in electrode buffer containing in addition 10% sodium dodecylsulfate and 10 mM N-ethylmaleimide. Before application on the gel, bromophenol blue is added as tracking dye. Gels are polymerized overnight under isobutanol. After allowing the protein to enter the gels slowly at a current of 10 mA per gel, the current is doubled and electrophoresis is continued for 16 hrs. After electrophoresis the position of the tracking dye is marked with India ink.

A mixture of polymers of bovine serum albumin is used for calibration purposes. The polymers are prepared as follows. A mixture of 100 mg bovine serum albumin and 60 mg dimethyl suberimidate in 7 ml 0.2 M triethanolamine buffer (pH 8.5) and 3 ml dimethyl sulfoxide is incubated for 1.5 hrs at 25°C, followed by another addition of 60 mg dimethylsuberimidate and another incubation for 1.5 hr at 25°C. The mixture is then dialyzed against distilled water, and lyophylized. The lyophilized protein is dissolved in sodium dodecylsulfate solubilization medium.

All gels are fixed and stained in 2.5% Coomassie blue 250 in methanol: acetic acid . water (4 : 1 . 5, by volume) at 60°C during 3 hrs. The gels are destained by diffusion of the unbound stain in water . acetic acid : methanol (8 . 1 :1, by volume) with several changes of destaining medium.

CHAPTER 3

PURIFICATION OF Na-K ATPase

3.1 Introduction

Na-K ATPase is a membrane bound protein (section 1.1.2). Since it is known to span the membrane (Kyte, 1975), it is an intrinsic membrane protein. This means that it is tightly bound to the membrane, and can only be separated from the other membrane components by drastic means such as detergent solubilization with the inherent danger of inactivation.

A pure Na-K ATPase preparation should have no other proteins present and show high catalytic activity. The latter condition implies that phospholipids must still be present, since complete delipidation inactivates the enzyme (section 1.8). Two approaches have been used for the purification of Na-K ATPase from a suitable membrane fraction:

a. Solubilization of the enzyme with a detergent and separation of the solubilized enzyme from other membrane components.
b. Solubilization of contaminating membrane proteins, leaving Na-K ATPase in the membrane as the only protein in a lipid environment. The extracted membranes are easily separated from the solubilized proteins.

It is, of course, desirable to choose a tissue with high Na-K ATPase activity. As mentioned by Bonting et al. (1961) there is considerable variation in the distribution of Na-K ATPase activity over various tissues. Highest activities on a dry weight basis are found in nerve tissue like brain and in tissues from certain secretory organs like kidney. Another tissue with high specific Na-K ATPase activity is the electroplax of the electric eel Electrophorus electricus.

Solubilization of the enzyme with detergents has been applied with success to microsomal preparations, which in some cases have first been treated with concentrated sodium iodide to remove ouabain-insensitive ATPase. This has been done with canine kidney medulla (Kyte, 1971; Lane et al., 1973), rectal gland of dogfish Squalus acanthias (Hokin et al., 1973), electroplax (Dixon and Hokin, 1974; Perrone et al., 1975) and pig brain

(Nakao et al., 1973). The non-ionic detergent Lubrol has been applied to solubilize the Na-K ATPase in all cases, exc. in that of canine kidney medulla, where the anionic detergent deoxycholate was used. Drawback of this approach is that the resulting preparations are labile, unless the detergent is removed in such a way that a lipid containing enzyme preparation is formed

Extraction of contaminating membrane proteins with a detergent has been applied successfully by Jørgensen (1974a). He solubilizes contaminating proteins from a microsomal preparation of kidney outer medulla with sodium dodecylsulfate (SDS). Then the extracted membranes are separated from the solubilized proteins on a sucrose density gradient. Pure preparations have been obtained with this method from kidney outer medulla of dog, sheep and rabbit (Jørgensen, 1974a) and of guinea pig (Hayashi et al., 1977) and also from duck nasal gland (Hopkins et al., 1976).

When we started our attempts to purify Na-K ATPase towards the end of 1973, the purification of this enzyme by means of the first method only had been described. The sources that were used were inaccessible to us on a regular basis (e g. electric eel electroplax, dogfish rectal gland or dog kidney outer medulla). Since fresh cattle brain was readily available to us and has a rather high Na-K ATPase activity we have used this source in our early experiments. The partial solubilization of the enzyme from NaI treated cattle brain microsomes by means of Lubrol had been reported (Kahlenberg et al., 1969). Our first approach has, therefore, been to isolate the enzyme from such a solubilized preparation by means of various techniques.

During this work the purification procedure based on selective extraction with SDS was published by Jørgensen (1974a). At first we have applied this method to cattle brain microsomal Na-K ATPase, which was not successful. Later we have applied the method to rabbit kidney outer medulla microsomes, and this has turned out to give good results when fresh (not frozen) rabbit kidney is used and when the outer medulla is carefully dissected out.

In this chapter the results of attempts to purify Na-K ATPase by different methods will be described.

3.2 Materials

The di-sodium salt of ATP is obtained from Boehringer Mannheim (W-Germany). Sepharose 4B, concanavalin A Sepharose 4B and sulphoethyl Sephadex are obtained from Pharmacia Fine Chemicals, Uppsala (Sweden) Sucrose is delivered by the British Drug House Ltd, Poole (England), while 1-O-methyl α-D Mannopyranoside is purchased from Calbiochem, San Diego, Ca. (USA). Phosphocellulose is supplied by Serva, Heidelberg (W-Germany) and sodium dodecylsulfate by Sigma, St. Louis, Mo. (USA) All other chemicals are obtained from Merck, Darmstadt (W-Germany) and are of the highest purity available. Lubrol (Cirrasol ALN-WF) is a gift from ICI Netherlands

3.3 Purification of Na-K ATPase after solubilization by Lubrol

3.3.1 Preparation of Lubrol solubilized Na-K ATPase

Cattle brains are obtained from the local slaughterhouse, and are transported in ice to the laboratory The cortex is taken and a microsomal fraction is prepared by differential centrifugation as described by Schwartz et al. (1962). Ouabain-insensitive Mg-ATPase activity is removed by NaI treatment as described by Nakao et al. (1965) The NaI treated microsomes are treated with Lubrol by the method of Kahlenberg et al. (1969). The specific Na-K ATPase activity of this solubilized preparation amounts to 48-60 μmol ATP hydrolyzed.mg protein^{-1}.hr^{-1}. Very little Mg ATPase activity is present (< 5% of total ATPase activity). This solubilized preparation is stable for several hours at 0°C When it is stored at -20°C in 50% (v/v) glycerol, there is a 25-60% loss of Na-K ATPase activity in 14 days

Further purification of the Na-K ATPase preparation has been attempted by iso-electric focussing, ion exchange chromatography and affinity chromatography.

3.3.2 Iso-electric focussing and ion exchange chromatography

During iso-electric focussing proteins are separated according to differences in iso-electric point. Atkinson et al. (1971) has reported the application of this technique to the purification of solubilized Na-K ATPase. In preliminary experiments with analytical iso-electric focussing

of Lubrol-solubilized Na-K ATPase on polyacrylamide gels we found a simple protein distribution pattern. Subsequently, preparative iso-electric focussing of solubilized Na-K ATPase in a glycerol gradient showed that all Na-K ATPase activity is concentrated in fractions with pH 4.6-5.0. Analysis of the protein distribution pattern in these fractions on SDS polyacrylamide gels demonstrated the presence of a number of different proteins, indicating a very impure preparation. The recovery of Na-K ATPase activity in these experiments was very low.

The low iso-electric point of Lubrol solubilized Na-K ATPase was then used in attempts to purify solubilized Na-K ATPase by ion exchange chromatography over phospho-cellulose and sulfoethyl-Sephadex columns. When applied to such columns at pH 4.5, Na-K ATPase should elute as one of the first proteins. However, no Na-K ATPase activity could be detected in the eluate of these columns, and hence this method was also abandoned.

The failure to achieve purification by iso-electric focussing may be due to the instability of Na-K ATPase at pH values below 5 (see section 4.3). It is also possible that Lubrol forms either mixed micelles of Na-K ATPase and other proteins, or single micelles of different proteins with about the same iso-electric points. The failure to achieve purification by ion exchange chromatography may be caused by delipidation with the attending inactivation of the enzyme.

3.3.3 Affinity chromatography of Lubrol solubilized Na-K ATPase

Affinity chromatography is based on the highly specific interaction of a protein with a (matrix bound) ligand and has been successfully employed in the purification of many enzymes and other proteins (for reviews see: Friedberg, 1971; Scouten, 1974). Agarose is widely used as a matrix to immobilize the ligand. The ligand can be coupled directly to the matrix. It can also be coupled by means of a bifunctional reagent, which acts as a spacer between matrix and ligand. The length of the spacer may influence the affinity of the ligand for the protein to be isolated (Cuatrecasas et al., 1968, Lowe et al., 1973).

For the purification of Na-K ATPase we have considered three types of ligands. ouabain, concanavalin A and ATP analogues. Although the preparation of matrix bound digoxin (a cardiac glycoside like ouabain) has been described (Okarma et al., 1972), no successful attempts of purification of

Na-K ATPase on such a gel have been reported. Hence, this approach has not been practised by us.

Concanavalin A binds reversibly with oligosaccharides containing a terminal α-D-glucopyranosyl or α-D-mannopyranosyl residue (Nicolson and Singer, 1971). Since Na-K ATPase has a glycoprotein subunit, which contains mannose (Perrone et al., 1975), it might bind to matrix bound concanavalin A, from which it would then be released upon addition of 1-O-methyl α-D-mannopyranoside to the eluent. Table 3.1 shows that Lubrol solubilized Na-K ATPase does bind to concanavalin A Sepharose (Pharmacia) and can be released at least in part by treatment with the carbohydrate. Unfortunately, upon

TABLE 3.1

BINDING OF LUBROL SOLUBILIZED Na-K ATPase TO CONCANAVALIN A SEPHAROSE

Gel	Addition	% Na-K ATPase activity in supernatant
Sepharose 4B	no	100
Con.A Sepharose	no	31
Con.A Sepharose	50 mM 1-O-methyl-α-D-mannopyranoside	73

The gel and Lubrol solubilized cattle brain cortex Na-K ATPase are equilibrated in a buffer containing 12 mM imidazole-HCl (pH 7.0), 0.4% (w/v) Lubrol, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 0.1 M NaCl with or without 1-O-methyl α-D-mannopyranoside. Equal volumes of gel and Na-K ATPase preparation are mixed for binding. Na-K ATPase activity is determined after 30 min at $0^{\circ}C$ in the supernatant and subsequent sedimentation of the gel. The activity is expressed as percent of the Na-K ATPase activity originally added. Sepharose 4B has been included as a control for aspecific binding.

columnchromatography of Lubrol solubilized Na-K ATPase over Con.A Sepharose no Na-K ATPase activity is eluted upon addition of 1-O-methyl α-D-mannopyranoside to the eluent. Hence, this method was abandoned.

A more extensive study was made of matrix bound ATP derivatives. Since the 6 aminogroup of the purine moiety and the β phosphate moiety are believed to be essential for the binding of ATP and ADP to Na-K ATPase (Hegyvary and Post, 1971; Nørby and Jensen, 1971), the nucleotide molecule must be attached to the matrix through another group. Affinity gels, containing ATP linked with its ribose group via a spacer to Sepharose 4B, have been synthesized according to Lamed et al. (1973). The amount of

phosphate per mg dry gel agreed with the value reported by these authors, and at least 80% of the nucleotide bound was ATP. In addition, affinity gels containing the nucleotide linked with its purine group via a spacer to Sepharose 4B have been prepared according to Anderton et al. (1973). Both types of affinity gels have been assayed for the binding of Lubrol solubilized Na-K ATPase, batch wise as well as in column chromatography. Unfortunately, no evidence for binding of Na-K ATPase or for elution of possibly bound Na-K ATPase from a column could be obtained.

The reason for the failure of our attempts to purify cattle brain Na-K ATPase by means of affinity chromatography may be that it is delipidated in the process and thus looses its activity (section 1.8). Marshall (1976) reports that Na-K ATPase is partly bound by concanavalin A Sepharose, and is eluted in a lipid-free form. Delipidation during affinity chromatography over a concanavalin A Sepharose column has also been reported by van Breugel et al. (1977) for rhodopsin. Although inactivation of Na-K ATPase through delipidation can in principle be reversed through reconstitution with suitable phospholipids, the elution process would then be very difficult to follow. This probably explains why no reports on purification of Na-K ATPase by affinity chromatography have appeared since the paper of Anderton et al. (1973).

3.4 Purification of Na-K ATPase by extraction of contaminating proteins

3.4.1 Preparation of microsomes

Rabbit kidneys, freshly obtained from a local packing plant, are stored in a buffer solution, containing 0.25 M sucrose and 30 mM histidine (pH 7.2) at $0^{\circ}C$. They can be thus stored for up to 15 hrs before dissection. The kidneys are cut in slices of 2-3 mm thickness On the cut surface, going from cortex to papilla, one can distinguish a brownish outer layer (cortex), a pink middle layer (outer medulla), and a white part (inner medulla with papilla). By careful dissection the outer medulla is obtained free from cortex and inner medulla, which is essential for successful purification.

The outer medulla is homogenized in ten volumes (w/v) of the storage buffer in a Potter-Elvehjem homogenizer (glass-Teflon) at 1500 rpm in five strokes. The resulting homogenate is centrifuged for 15 min at $6000g_{max}$.

While saving the supernatant, the pellet is rehomogenized in the same volume of buffer and centrifuged again for 15 min at $6000g_{max}$. The two supernatants are combined and centrifuged for 30 min at $48,000g_{max}$. The 48,000g pellet, which is called the microsomal fraction, is homogenized in the same buffer and stored at $-20^{o}C$. All operations are carried out on ice or at $0-4^{o}C$, and all centrifugations are performed in a Sorvall RC 2B centrifuge in a Sorvall SS 34 rotor.

The specific Na-K ATPase activities in the microsomal fraction have been determined, when obtained from fresh as well as from frozen kidneys (Table 3.2). These results indicate that freezing the kidneys before

TABLE 3.2

ATPase ACTIVITIES IN MICROSOMAL FRACTIONS OF RABBIT KIDNEY OUTER MEDULLA

Microsomal preparation	Na-K ATPase	Mg ATPase
frozen kidney	21	24
frozen kidney, detergent treated	63	20
fresh kidney	60	80
fresh kidney, detergent treated	240	60

The ATPase activities are expressed as μmol ATP.mg protein^{-1}.hr^{-1}.

dissection and homogenization causes a great loss of activity. Hence, we have subsequently only used unfrozen kidneys.

3.4.2 Extraction with sodium dodecylsulfate and Sucrose density centrifugation

Microsomes are incubated in a medium containing 25 mM imidazole-HCl (pH 7.4), 5 mM ATP, 2 mM EDTA, 0.58 mg.ml^{-1} SDS. The detergent is added after addition of 1.4 mg.ml^{-1} microsomal protein and mixing. The mixture (final volume 100 ml) is incubated for 1 hr at $20^{o}C$. The incubated preparation is applied on a sucrose density gradient. The gradient is prepared in an IEC B30 zonal gradient rotor. It is a linear gradient of 15-40% (w/v) sucrose over a cushion of 50% (w/v) sucrose. The sucrose solutions contain 1 mM EDTA and 25 mM imidazole-HCl (pH 7.5). After sealing the rotor and establishing a vacuum in the rotor chamber of the IEC B60 ultracentrifuge, the rotor is accelerated to 47,000 rpm. It is then centrifuged for 2 hrs

at this speed (150,000g_{max}) at 4°C. After braking the rotor to 3000 rpm, it is emptied dynamically and the gradient is divided into 15 ml fractions. The 254 nm absorbance is continuously monitored. The fractions, which contain Na-K ATPase activity, are diluted 1 : 1 with a buffer containing 2 mM ATP, 1 mM EDTA, 25 mM imidazole-HCl (pH 7.5). This mixture is centrifuged for 60 min at 44,000 rpm in an IEC A 211 rotor (200,000g_{max}). The resulting pellets, which contain the Na-K ATPase activity, are rehomogenized in 1 mM EDTA, 25 mM imidazole-HCl (pH 7.5) and stored at 0°C. They can also be stored frozen at -20°C, when 250 mM sucrose is added to the EDTA-imidazole solution.

The yield from one gradient can be doubled by extracting the double amount of microsomes (280 mg protein) with SDS in a double volume (200 ml) of the above SDS-containing buffer. After 1 hr incubation at 20°C the mixture is centrifuged for 30 min at 30,000 rpm (100,000g_{max}) in an IEC A 211 rotor. The resulting pellets are rehomogenized in an aliquot of the supernatant (final volume 50 ml). This homogenate is loaded on a sucrose density gradient, and further treated as described above.

Fig. 3.1 shows the results of a zonal gradient run. The Na-K ATPase activity is recovered in a fraction with a density of 1.12-1.14 g.ml^{-1}. The total amount of Na-K ATPase activity in the peak fractions is about 60% of the activity applied on the gradient. The specific activities of the final preparation range from 1000-2200 µmol ATP hydrolyzed.mg $protein^{-1}$. .hr^{-1}. The purity on a protein basis is 90% (see section 4.2). The protein and lipid composition of the enzyme preparation is described in section 4.2.

3.4.3 Discussion

It is possible to obtain a highly active and highly pure preparation of Na-K ATPase by extraction of contaminating proteins from the membrane according to the method of Jørgensen (1974a). It appears that the success of this method is due to the fact that peripheral membrane proteins bind SDS more rapidly than do intrinsic membrane proteins (Helenius and Simons, 1975). A prerequisite for success in the purification of Na-K ATPase with this method is that the enzyme is the dominant intrinsic membrane protein in the microsomal preparation. The choice of the tissue is, therefore, critical. The procedure does not work well with cattle brain microsomes, whether or not they have been treated with NaI before extraction. Maximal

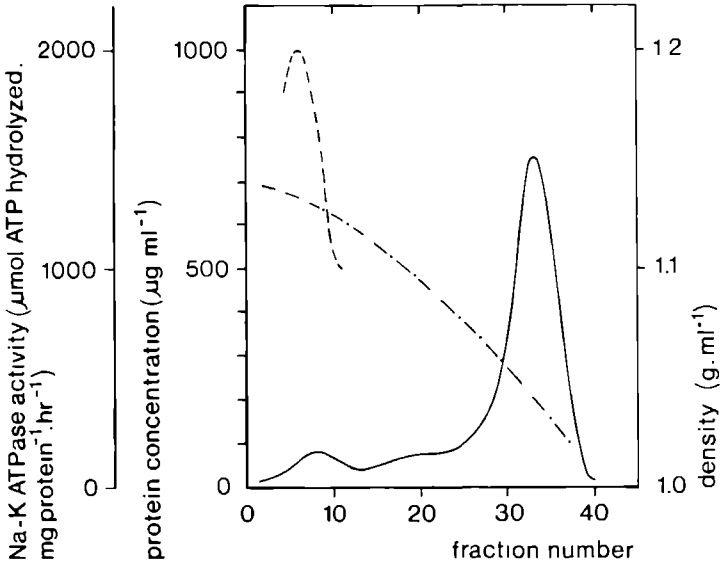


Fig. 3.1 Preparative Sucrose density centrifugation of SDS extracted kidney outer medulla microsomes. The experiment has been performed as described in section 3.4.2. 140 mg protein are applied to the preparative sucrose density gradient. Na-K ATPase activity (-----) and Protein concentration (———) are determined as described in sections 2.3,(method I) and 2.9 respectively. Density of the gradient fractions (-·-·-) has been calculated after the determination of the refractive index.

activities of only 200 µmol ATP hydrolyzed.mg protein^{-1}.hr^{-1} have been obtained. No single Na-K ATPase containing peak fraction is found after sucrose density centrifugation of the extracted cattle brain microsomes. The low specific activity and the distribution of Na-K ATPase over all fractions of the sucrose density gradient have led us to abandon this material as a source for the purification of the enzyme.

With rabbit kidney excellent results are obtained. When using frozen rabbit kidneys the microsomal fraction of outer medulla shows only a quarter of the specific Na-K ATPase activity that is shown in a similar preparation made from fresh rabbit kidneys. This is found for preparations made from kidneys, that have been frozen before dissection of the outer medulla, as well as for dissected fresh kidney outer medulla that has been frozen before homogenization and fractionation. After SDS extraction and sucrose density centrifugation the specific Na-K ATPase activity of the

purified preparation from frozen kidneys never exceeds 900 μmol ATP hydrolyzed.mg protein^{-1}.hr^{-1}. On SDS gels many proteins, which do not belong to the Na-K ATPase complex (see section 1.7), are observed in this fraction

When, however, fresh rabbit kidneys are used as starting material, the purification method of Jørgensen (1974a) gives a highly active and highly pure preparation, which is completely reproducible. Dissection is critical. when cortex or inner medulla are present in the dissected outer medulla, the resulting specific activity is much lower. Further characteristics of this purified Na-K ATPase preparation will be given in the next chapter (chapter 4).

CHAPTER 4

CHARACTERISTICS OF PURIFIED Na-K ATPase

4.1 Introduction

In this chapter the main characteristics of the purified Na-K ATPase preparation from rabbit kidney outer medulla, prepared as described in section 3.4, will be described. Since previously most parameters of the enzyme have been determined in crude preparations, it has seemed desirable to determine these parameters again in the purified preparation. Knowledge of these parameters is important for the evaluation of the modification experiments to be described in chapters 5-9.

Most of the assays have been reported previously, but have been adapted for a pure preparation where necessary. These assays are described in chapter 2. The parameters of the K^+ stimulated 4-nitrophenylphosphatase assay are determined in more detail, because this activity is supposed to represent a partial reaction of the Na-K ATPase reaction (section 1.4.5). Effects of chemical modification on this partial activity may be helpful to elucidate the mechanism of action of the enzyme. Finally, some general remarks will be made about the purity and stability of the purified enzyme preparation.

4.2 Protein and lipid composition

The Na-K ATPase molecule consists of two subunits with different molecular weights, a 100,000 MW catalytic subunit and a 50,000 MW glycoprotein subunit (section 1.7). Thus a pure Na-K ATPase preparation should only show protein bands deriving from these subunits upon SDS gel electrophoresis. In fig. 4.1 the protein composition of a purified Na-K ATPase preparation after SDS gel electrophoresis on a 8% (total) acrylamide gel according to the Laemmli method (section 2.9.2) is presented. For comparison the protein composition of the microsomes from rabbit kidney outer medulla used for its preparation is also included. Whereas the microsomal preparation displays many protein bands, the purified preparation shows only two

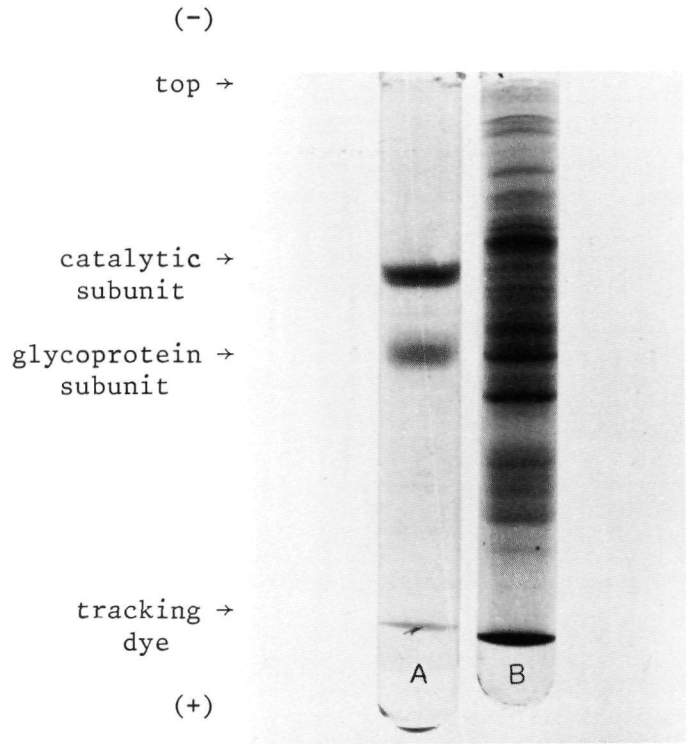


Fig. 4.1 Gel electrophoretic pattern of purified Na-K ATPase. A purified rabbit outer medulla Na-K ATPase preparation (A) and crude rabbit kidney outer medulla microsomes (B) have been subjected to polyacrylamide SDS gel-electrophoresis according to the Laemmli method (section 2.9.2). The gels have an 8% total acrylamide content. The specific Na-K ATPase activity of the pure enzyme preparation amounts to 2000 μmol ATP hydrolyzed.mg protein^{-1}.hr^{-1}.

bands with apparent molecular weights of 95,000 and 43,000. The low molecular weight protein is a glycoprotein, since it shows a periodic acid-Schiff stain. Hence, the molecular weight derived from gel electrophoresis may be too large. A more reliable value can be obtained by determining the molecular weight on gels with various acrylamide concentrations and extrapolating the observed values to the apparent molecular weight in a 100% acrylamide gel (Segrest and Jackson, 1972). Fig. 4.2 illustrates the results of such an experiment, indicating that the apparent molecular weight of the glycoprotein subunit is 43,000.

There is also a band staining with Coomassie blue at the position of the tracking dye. Such a band has previously been reported by other authors

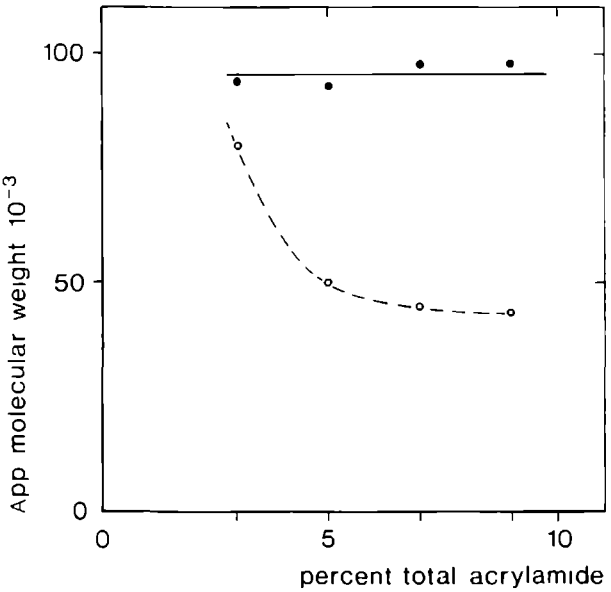


Fig. 4.2 Apparent molecular weights of Na-K ATPase subunits in polyacrylamide gel electrophoresis. Apparent molecular weights (—•—, catalytic subunit and ---o---, glycoprotein subunit) have been determined in gels with varying polyacrylamide content, but with a constant acrylamide/bisacrylamide ratio. Gel electrophoresis is performed by the Laemmli method (section 2.9.2).

(section 1.7). It has been suggested that it consists of glycolipids or phospholipid, but no definite knowledge about its chemical identity and origin exists.

Since no other protein bands are visible in SDS gel electrophoresis beside the 95,000 and 43,000 subunits, the enzyme preparation must be more than 90% pure on protein basis. In subsequent chapters, we shall refer to these entities as the 100,000 and 50,000 subunits, as they are usually referred to in the litterature.

The preparation still contains phospholipids, the composition of which (de Pont et al., to be published) is given in table 4.1. Both the presence and composition of the phospholipids and electron microscopic observations (Prof.Dr. E.L. Benedetti, Paris, private communication) indicate that the preparation still consists of membrane fragments (cf. Maunsbach and Jørgensen, 1974).

Table 4.1

PHOSPHOLIPID CONTENT AND COMPOSITION OF PURIFIED Na-K ATPase FROM RABBIT KIDNEY OUTER MEDULLA

	($\mu g.mg\ protein^{-1}$)	(moles phospholipid per mole Na-K ATPase)
Phospholipid content	33.1 ± 2.4	267 ± 19
	Phospholipid composition	
Phospholipid	(% of total phospholipids)	(moles per mole Na-K ATPase)*
Sphingomyelin	17.9 ± 0.6	48 ± 4
Phosphatidylcholine	35.6 + 0.7	95 ± 7
Phosphatidylserine	13.1 + 0.9	35 ± 4
Phosphatidylinositol	5.5 ± 0.3	15 ± 2
Phosphatidylethanolamine	27.9 ± 1.0	74 ± 6

*Calculated from phospholipid composition and lipid-P content, assuming a molecular weight of 250,000 for Na-K ATPase.
The results are presented as averages with standard errors for 15 preparations. (de Pont et al., to be published).

4.3 Na-K ATPase activity

The Na-K ATPase activity is determined as described in section 2.3 (method I). The activity of the purified Na-K ATPase preparations ranges from 800 to 2300 μmol ATP hydrolyzed.mg protein $^{-1}$.hr^{-1}, with an average of 1638 (SE 120) (Table 4.2). There is no detectable ouabain-insensitive activity in these preparations. Assuming a 250,000 MW and 90% purity of the enzyme, this would give a turnover number of 7600 min^{-1}, in good agreement with the value of 8800 min^{-1} determined by Jørgensen (1974b) from ouabain binding experiments.

The pH for optimal Na-K ATPase activity in the purified Na-K ATPase preparation amounts to 7.5 (fig. 4.3). This agrees well with the optimal pH value for rat kidney homogenate (pH 7.4) reported by Bonting (1970, p. 267).

The inhibition of the Na-K ATPase activity by ouabain has also been determined in the purified preparation. As shown in fig. 4.4, the Na-K

Table 4.2

SPECIFIC Na-K ATPase ACTIVITY, Na^+ DEPENDENT PHOSPHORYLATION BY ATP AND TURNOVER NUMBER OF TWELVE PURIFIED Na-K ATPase PREPARATIONS

specific activity	phosphorylation	turnover number
μmol ATP hydrolyzed. .mg protein^{-1}.hr^{-1}	pmol P bound. .mg protein^{-1}	10^3 min^{-1}
769	1084	11.8
978	982	16.6
1429	1368	17.5
1443	1956	12.2
1725	1709	16.8
1733	1813	15.9
1757	1723	17.0
1819	2211	13.7
1844	1884	16.3
1950	1808	18.0
1960	1813	20.0
2254	1848	20.3
av. 1638 ± 120	1683 ± 100	16.3 ± 0.8

Specific Na-K ATPase activities in twelve purified rabbit kidney outer medulla preparations (first column), determined by method I of section 2.3, are ranked in order of increasing activity. The Na^+ dependent phosphorylation by ATP of each preparation has been determined at 0^oC as described in section 2.5 (second column). The turnover number has been calculated from the data in the first two columns, assuming that all active centers have been phosphorylated by one molecule of ATP each. There is a significant positive correlation between the values of column 1 and 2 ($P < 0.04$) and also between those of columns 3 and 1 ($P < 0.02$), according to the rank correlation method of Kendall (Mann, 1945).

ATPase activity is inhibited by ouabain with a $\bar{p}I_{50}$ of 5.8 and full inhibition occurs at 0.3 mM. It also shows the biphasic effect, earlier reported by Bonting in crude preparations (1970, p. 268)

In accordance with the observations of Jørgensen (1974a), the enzyme activity of the purified preparation is stable for several days, when stored unfrozen at 0^oC in 25 mM imidazole-HCl (pH 7.5), 1 mM EDTA. The preparation can be stored for several months in 25 mM imidazole-HCl

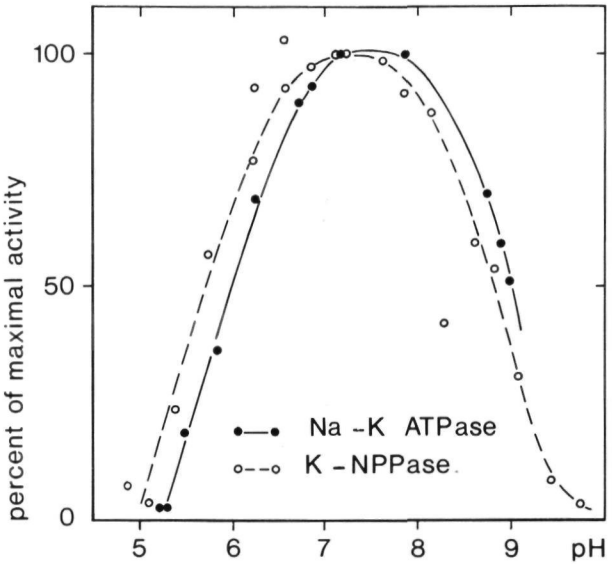


Fig. 4.3 Na-K ATPase and K-NPPase activities as a function of pH. Na-K ATPase (—•—) and K-NPPase (---o---) activities in purified rabbit kidney outer medulla preparations are determined as described in sections 2.3 (method I) and 2.7. Instead of the imidazole buffer, various Tris-maleic acid mixtures (sum of both concentrations kept at 100 mM) are used to obtain the desired pH values.

(pH 7.5), 1 mM CDTA, 250 mM sucrose at -20°C. The latter storage method has been used routinely.

The stability of the preparation at 37°C depends strongly on the pH of the medium. In fig. 4.5 the residual Na-K ATPase activity after 1 hr incubation in media with various pH values is shown. The activity is resistent against incubation at pH values over pH 6.0, but is rapidly lost in media at lower pH. This is an irreversible loss, since the subsequent assay takes place in a medium of pH 7.4.

4.4 Na^+ dependent phosphorylation by ATP

The Na-dependent phosphorylation capacity with ATP of twelve purified Na-K ATPase preparations has been determined at 0°C by the method described in section 2.5. The results are listed in Table 4.2 (second column). The values range from 980 to 2211 pmol P bound per mg protein with an average

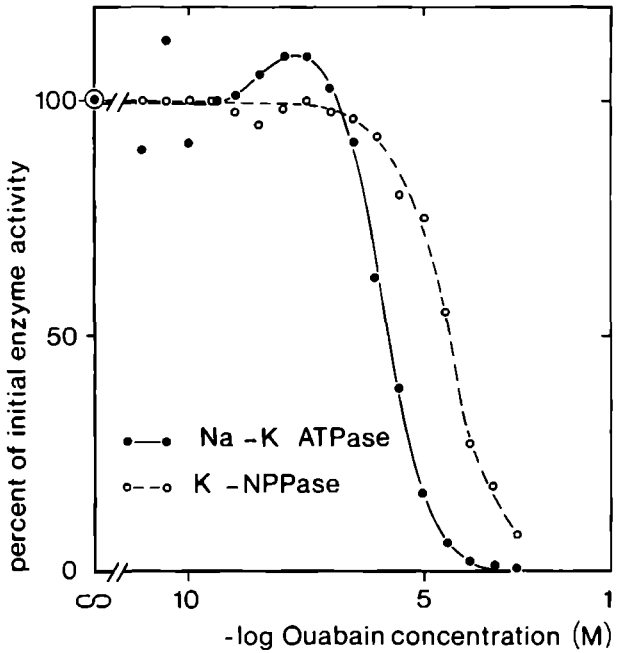


Fig. 4.4 Inhibition of Na-K ATPase and K-NPPase activities by ouabain. The Na-K ATPase (—•—) and K-NPPase (---o---) activities of purified Na-K ATPase preparations from rabbit kidney outer medulla are determined as described in sections 2.3 (method I) and 2.7. Ouabain is added to the media containing 10 mM KCl in final concentrations as indicated.

of 1683 (SE 100). There is a positive correlation with the specific Na-K ATPase activities in column 1, indicating that the number of active enzyme molecules present per mg protein increases with the specific activity.

From the values for the specific activity and the phosphorylation capacity the turnover number can also be calculated, assuming that all active centers have been phosphorylated by one molecule of ATP each. The values range from 11,800 to 20,300 min^{-1} with an average of 16,300 (SE 770). There is also a positive correlation between these values and the specific activity ($P = 0.02$), which indicates that preparations with high numbers of active enzyme molecules have more activity than accounted for simply on the basis of these numbers. This value for the turnover number is considerably higher than that calculated from the Na-K ATPase activity (7600 min^{-1}, section 4.3) and that reported by Jørgensen (1974b) from ouabain binding

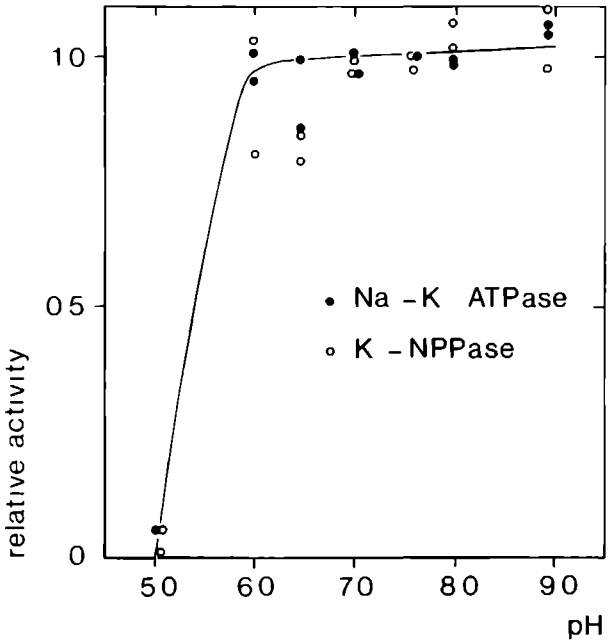


Fig. 4 5 Stability of Na-K ATPase and K-NPPase activities upon incubation at varying pH. Purified rabbit kidney outer medulla Na-K ATPase is incubated for 60 min at 37°C in 25 mM imidazole-HCl at the indicated pH value. After incubation the residual Na-K ATPase activity (•) is determined as described in section 2.3 (method I) and K-NPPase activity (o) as described in section 2.7. The relative activity is expressed with respect to the mean residual activity at pH 7.4.

experiments. This is probably due to the fact that rapid dephosphorylation occurs during phosphorylation of the enzyme, which will lead to an underestimation of the number of active centers

4.5 Phosphorylation by inorganic phosphate

Binding of P_i to the enzyme requires Mg^{2+} ions, and the stability of the enzyme-phosphate complex is enhanced by previous binding of ouabain (Lindenmayer et al., 1968, Post et al., 1975). To characterize the phosphate binding process, we have determined the maximal phosphate incorporation in pmol mg protein^{-1} and the dissociation constant K_{diss} (in µM) of the enzyme-phosphate complex with or without preincubation with ouabain.

Ouabain preincubation is performed in a medium containing 5 mM $MgCl_2$, 10 mM imidazole-HCl (pH 7.0), 0.1 mM ouabain and 0.3 mg/ml protein in a final volume of 450 µl for 30 min at 20°C. Phosphorylation of the enzyme is performed as described in section 2 6. Various concentrations of P_i (1-100 µM) are used for phosphorylation. K_{diss} and maximal P_i incorporation are determined from a Scatchard plot of the results.

The figures in table 4.3 indicate, somewhat surprisingly, that the

Table 4.3

PHOSPHORYLATION BY INORGANIC PHOSPHATE

preincubation	+ ouabain	- ouabain
K_{diss} (µM)	25 + 3	34 ± 2
maximal phosphate incorporation (pmol P mg protein^{-1})	2390 + 30	2370 ± 40

Phosphorylation of Na-K ATPase preparations from purified rabbit kidney outer medulla is performed as described in section 2.6. Preincubation with ouabain is described in section 4 5. Specific Na-K ATPase activity of the enzyme preparation is 1300 µmol ATP hydrolyzed.mg protein^{-1}.hr^{-1}.

effect of prior binding of ouabain on K_{diss} is rather small. This cannot be due to a low affinity of renal Na-K ATPase for ouabain, since both the Na-K ATPase and K-NPPase activities are inhibited at this concentration, and preincubation with ouabain under these conditions does affect the phosphorylation by 4-nitrophenylphosphate.

4.6 Na^+ stimulated ATPase activity

In crude Na-K ATPase preparations a Na^+ stimulated ATPase activity at low substrate concentration can be determined, which is inhibited by K^+ ions as well as by high ouabain concentrations (Neufeld and Levy, 1969; Post et al., 1972). This activity is also observed in purified Na-K ATPase preparations from rabbit kidney outer medulla. The specific Na^+ stimulated ATPase activity at 1 µM ATP concentration amounts to 21.7 µmol ATP hydrolyzed.mg protein^{-1}.hr^{-1}. This is 0.2% of the specific Na-K ATPase activity of the preparations used for this assay. There is a positive correlation between these two activities. The Na^+ stimulated ATPase activity is completely inhibited after addition of 10^{-3} M ouabain to the assay medium.

4.7 K^+ stimulated 4-nitrophenylphosphatase activity

There is a linear relationship between the K-NPPase activity and the Na-K ATPase activity of purified Na-K ATPase preparations of rabbit kidney outer medulla (fig. 4.6). This finding, combined with the gel electro-

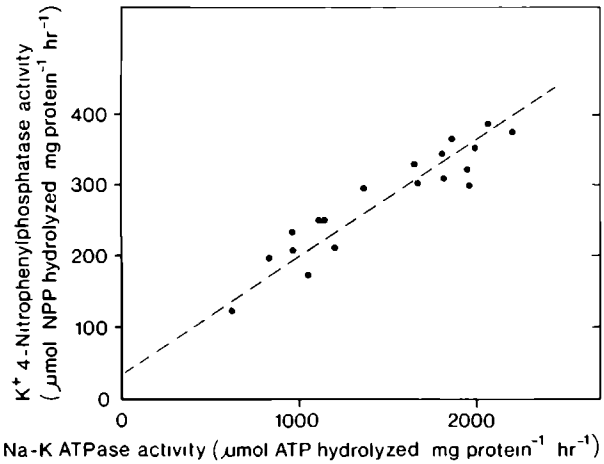


Fig. 4.6 Relation between K-NPPase and Na-K ATPase activities in various purified enzyme preparations. Na-K ATPase activity is determined as described in section 2.3 (method I), K-NPPase activity as described in section 2.7. The dashed line through the experimental points is the best fitting line, determined with the method of least squares. The correlation coefficient for this line is 0.93.

phoretic purity of the best preparations (fig. 4.1), indicates that both activities derive from the same enzyme. K-NPPase activity may thus be considered to be a partial activity of the Na-K ATPase system, representing the final dephosphorylating step of the ATP hydrolysis reaction (section 1.4.5).

The optimal pH of the K-NPPase activity (pH 7.3) is slightly lower than that of the Na-K ATPase activity (pH 7.5; fig. 4.3). The K-NPPase activity is inhibited by ouabain, but the pI_{50} value of 4.5 is lower than that for the Na-K ATPase activity (5.8; fig. 4.4). Ouabain does not have a biphasic effect in this case, as it does on the Na-K ATPase activity. The

20-fold higher ouabain concentration needed for 50% inhibition may be due to the absence of Na^+ ions and ATP in the K-NPPase assay medium. The relatively high K^+ concentration in this medium may also play a role, since K^+ ions are known to antagonize ouabain binding and inhibition.

Maximal activity, in the presence of 10 mM KCl, 1 mM CDTA and 6 mM $MgCl_2$ at pH 7.5, is obtained at 5 mM 4-nitrophenylphosphate, while half-maximal activity is reached at 0.5 mM (fig. 4.7).

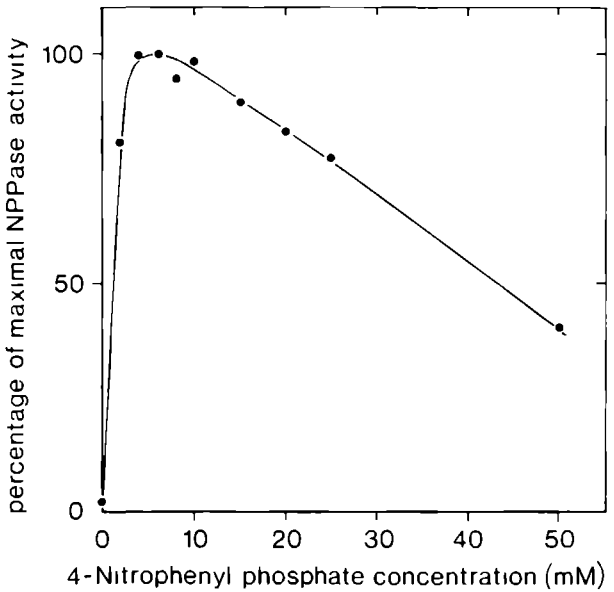


Fig. 4.7 K-NPPase activity at various concentrations of 4-nitrophenyl-phosphate. K-NPPase activity in purified rabbit kidney outer medulla preparations is determined as described in section 2.7.

The effects of monovalent cations on the NPPase activity in Na-K ATPase preparations are shown in fig. 4.8. The greatest effect is seen with K^+, the effectiveness decreasing in the order: $K^+ > Tl^+ > Li^+ > Na^+ = 0$. The activity decreases when the ionic strength of the reaction medium is increased by addition of $choline^+$ in the presence of 10 mM K^+. Na^+ ions inhibit rather than activate the hydrolysis of 4-nitrophenylphosphate (fig. 4.9, and table 4.4).

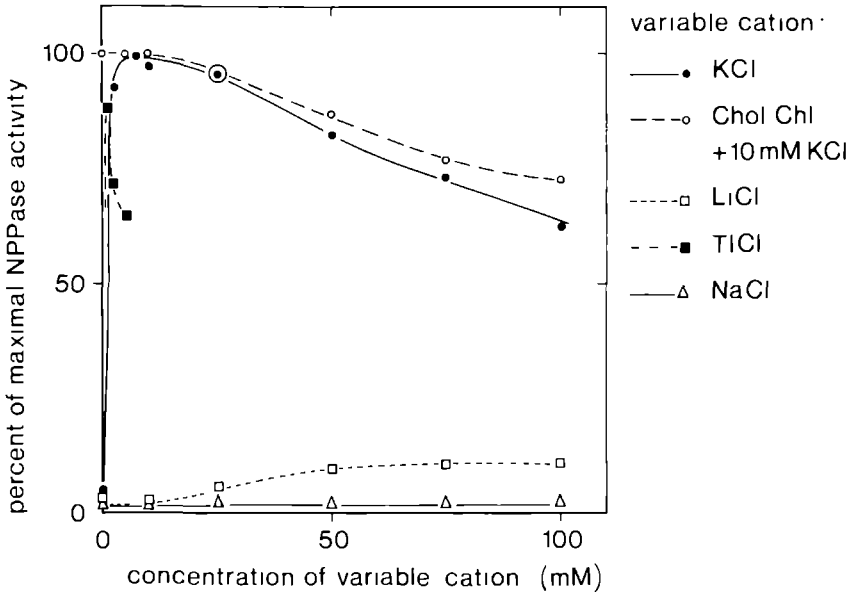


Fig. 4.8 Effects of various monovalent cations on p-nitrophenylphosphatase activity. The NPPase activity of purified Na-K ATPase preparations from rabbit kidney outer medulla is determined as described in section 2.7, except that KCl is omitted from the medium. Monovalent cations (— • — K^+ -·-■-·- Tl^+, ···□··· Li^+, —Δ— Na^+) are added as chloride salts in the stated concentrations. Choline chloride (---o---) is added in addition to a constant concentration of 10 mM KCl.

The presence of a divalent cation is required, Mg^{2+} being the most potent cation. As shown in fig. 4.10, Mn^{2+} can replace Mg^{2+} in part, but Ca^{2+} cannot replace Mg^{2+}. The optimal Mg^{2+}/substrate ratio, which for Na-K ATPase is 1, is less than 1 for K-NPPase at substrate concentrations above 2 mM (fig. 4.11). This may indicate the existence of an inhibitory Mg^{2+} binding site of low affinity.

In the presence of 20 mM Na^+ and at maximally stimulating K^+ concentration (10 mM), the addition of ATP (0.1 mM) has a slightly inhibitory effect (Table 4.4). At low K^+ concentration (0.1 mM) the addition of ATP and Na^+ is stimulatory, ADP and Adenylyl imidodiphosphate (AMPPNP) are slightly inhibitory (Table 4.4). This suggests that phosphorylation of the enzyme by ATP may be involved in the stimulation of the K-NPPase activity. In the absence of Na^+, which precludes phosphorylation, ATP does not

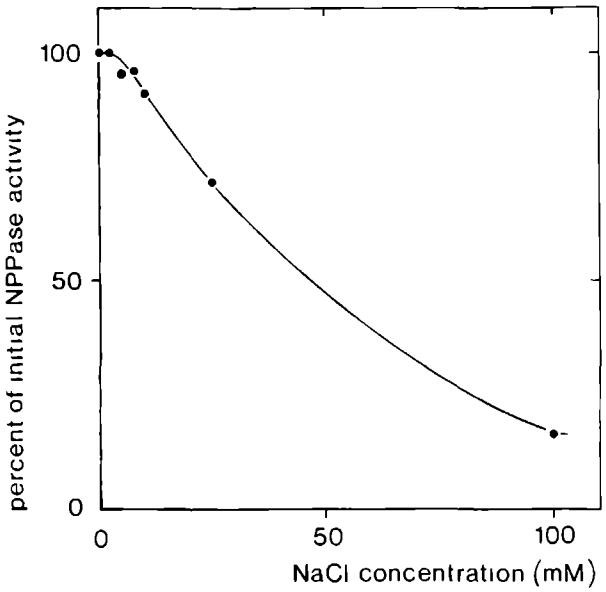


Fig. 4.9 Effect of Na^+ ions on K-NPPase activity. The K-NPPase activity is determined in purified Na-K ATPase preparations from rabbit kidney outer medulla as described in section 2.7. NaCl is added to the reaction medium in the stated concentrations.

enhance K-NPPase activity. This phenomenon may be due to the fact that at low K^+ concentration the enzyme is partly in an E_1 conformation, which conformation is not involved in the K-NPPase reaction. Phosphorylation of the enzyme by ATP would convert the E_1 conformation into an E_2 conformation, which is involved in the K-NPPase activity.

From these findings it is clear that maximal K-NPPase activity is obtained in the presence of 10 mM KCl, 1 mM CDTA, 6 mM $MgCl_2$ and 5 mM NPP at a pH of 7.5. The buffer concentration is set at 10 mM imidazole-HCl because of the ionic strength effects on K-NPPase activity. The composition of the K-NPPase activity assay medium is based on these considerations.

4.8 Phosphorylation by (^{32}P)-4-nitrophenylphosphate

Previously phosphorylation of the Na-K ATPase system by (^{32}P)-4-nitrophenylphosphate (abb. (^{32}P)NPP) has been performed in crude preparations (Inturrisi and Titus, 1970, Robinson, 1971a and b). These experiments have

Table 4.4

EFFECTS OF NUCLEOTIDES ON K-NPPase ACTIVITY

Additions	K-NPPase activity (% of control activity)
I 10 mM KCl in K-NPPase medium	
none	= 100
20 mM NaCl	78
20 mM NaCl + 0.1 mM ATP	62
II. 0.5 mM KCl in K-NPPase medium	
none	= 100
20 mM NaCl	134
20 mM NaCl + 0.1 mM ATP	300
20 mM NaCl + 0.1 mM ADP	60
20 mM NaCl + 0.1 mM AMPPNP	100
0.1 mM ATP	45

The K-NPPase activity is determined in purified Na-K ATPase preparations from rabbit kidney outer medulla, as described in section 2.7, in the presence of 10 mM or 0.5 mM KCl. Effects of 20 mM NaCl and 0.1 mM ATP are maximal, higher or lower concentrations are less stimulating or even inhibitory. When addition of nucleotides or NaCl leads to inhibition, no stimulating effect is found at any concentration up to 100 mM NaCl or 2.5 mM nucleotide.

now been repeated with the purified Na-K ATPase preparation according to the method described in section 2.8. Preincubation with ouabain (0.12 mM) has been carried out in 25 mM imidazole-HCl (pH 7.5) with or without 1.4 mM $MgCl_2$. In the phosphorylation medium these concentrations are lowered to 1 mM $MgCl_2$ and 0.09 mM ouabain. The phosphorylation conditions have been varied (figs. 4.12 and 4.13).

Phosphorylation by NPP requires the presence of Mg^{2+} ions (fig. 4.12, bar A vs bar C). Preincubation of the enzyme with ouabain plus Mg^{2+} results in higher phosphorylation levels than without such preincubation (cf. fig. 4.12, C and D vs. fig. 4.13, A and B). Higher phosphorylation levels are reached in one min at 37°C than at 0°C (cf. fig. 4.12, E and F vs. C and D). Addition of K^+ ions to a Mg^{2+}-containing phosphorylation medium increases the phosphorylation level (cf. fig. 4.12, D vs. C and

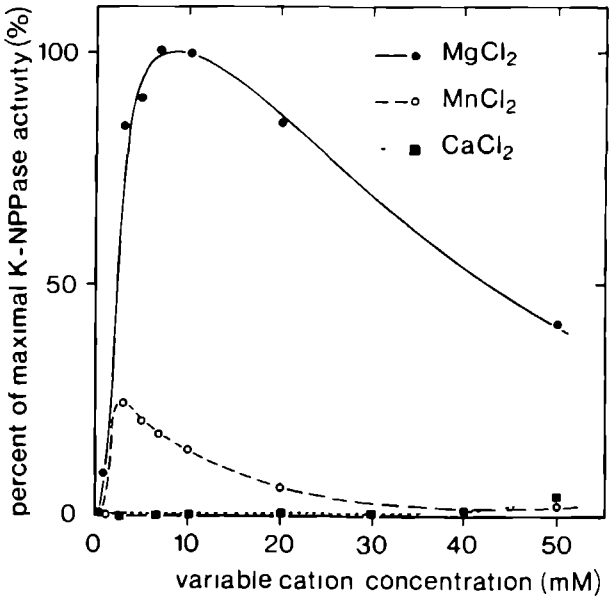


Fig. 4.10 Effects of divalent cations on K-NPPase activity. The K-NPPase activity of purified Na-K ATPase preparations from rabbit kidney outer medulla is determined as described in section 2.7. Instead of 6 mM $MgCl_2$, divalent cations (——●—— Mg^{2+}, ---o--- Mn^{2+}, ··■· · Ca^{2+}) are added to the incubation medium as their chloride salts in the stated concentrations.

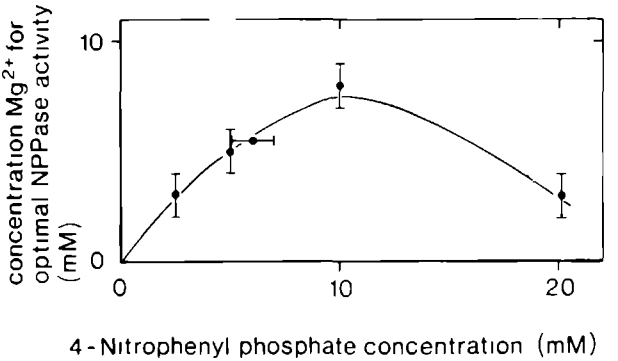


Fig. 4.11 Ratio of Mg^{2+} to NPP for optimal K-NPPase activity. K-NPPase activity of Na-K ATPase preparations from rabbit kidney outer medulla has been determined as described in section 2.7. Various concentrations of NPP have been used, and the Mg^{2+} concentration is varied at each NPP concentration (or vice versa, see fig. 4.7). The Mg^{2+} concentration giving optimal activity is plotted against the corresponding NPP concentration. Mg^{2+} concentrations are corrected for binding to CDTA by substracting 1 mM from the total $MgCl_2$ concentration.

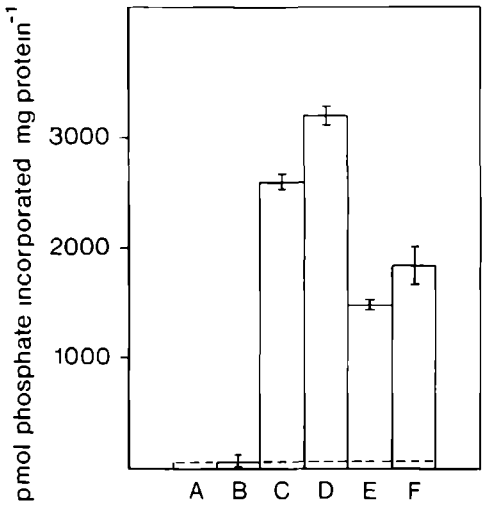


Fig. 4.12 Phosphorylation by (^{32}P)-4-nitrophenylphosphate after preincubation with ouabain. The phosphorylation of purified Na-K ATPase preparations from rabbit kidney outer medulla is performed as described in section 2.8. Preincubation and phosphorylation conditions are varied as follows:

Bar	preincubation medium	phosphorylation medium	temp.
A	0.12 mM ouabain	ouabain	37°C
B	0.12 mM ouabain	ouabain, 5 mM KCl	37°C
C	0.12 mM ouabain + 1.4 mM $MgCl_2$	ouabain, $MgCl_2$	37°C
D	0.12 mM ouabain + 1.4 mM $MgCl_2$	ouabain, $MgCl_2$, 5 mM KCl	37°C
E	0.12 mM ouabain + 1.4 mM $MgCl_2$	ouabain, $MgCl_2$	0°C
F	0.12 mM ouabain + 1.4 mM $MgCl_2$	ouabain, $MgCl_2$, 5 mM KCl	0°C

Preincubation is carried out for 30 min at 20°C. Phosphorylation is carried out for 1 min. All values have been corrected for blank phosphorylation, the dashed line indicating the variation in this correction.

F vs. E; fig. 4.13, B vs. A, but not F vs. E). Addition of ATP and Na^+ ions in concentrations, which enhance the K-NPPase activity (see table 4.4), reduces the phosphorylation level (cf. fig. 4.13, C vs. A and D vs. B). In conclusion it can be said that the best conditions for phosphorylation by (^{32}P) NPP are: preincubation with 0.12 mM ouabain and 1.4 mM $MgCl_2$ for 30 min at room temperature, followed by phosphorylation for 1 min at 37°C in a medium containing 0.09 mM ouabain, 1 mM $MgCl_2$, 2 mM (^{32}P) NPP, and 5 mM

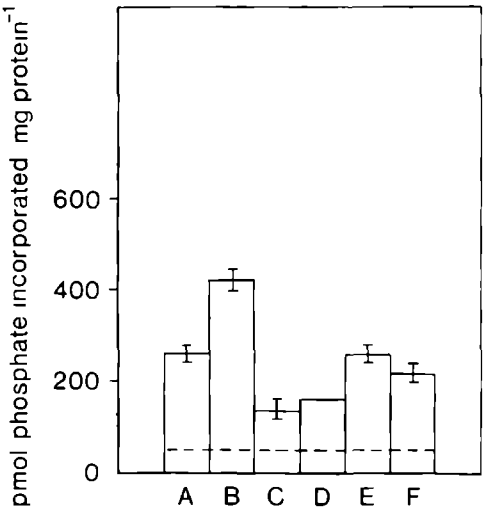


Fig. 4.13 Phosphorylation by (^{32}P)-4-nitrophenylphosphate. Phosphorylation of purified Na-K ATPase preparations from rabbit kidney outer medulla is performed as described in section 2.8. Phosphorylation conditions are varied as follows:

Bar		temperature
A	1 mM $MgCl_2$	37°C
B	1 mM $MgCl_2$, 5 mM KCl	37°C
C	1 mM $MgCl_2$, 0.1 mM ATP, 20 mM NaCl	37°C
D	1 mM $MgCl_2$, 5 mM KCl, 0.1 mM ATP, 20 mM NaCl	37°C
E	1 mM $MgCl_2$	0°C
F	1 mM $MgCl_2$, 5 mM KCl	0°C

Phosphorylation is carried out for 1 min at the indicated temperature. All values have been corrected for blank phosphorylation, the dashed line indicating the variation in this correction.

KCl at pH 7.5.

Since the enzyme can also be phosphorylated by $^{32}P_i$ (section 4.6), it is necessary to make sure that the ^{32}P incorporated upon incubation with (^{32}P) NPP does not derive from $^{32}P_i$ enzymatically split off from the substrate or present as a contamination of the substrate. The first possibility has been investigated by determining in parallel the enzymatic release of P_i from 4-nitrophenylphosphate and the phosphorylation of the enzyme at 0°C in the presence of Mg^{2+} ions (fig. 4.14). The initial rate of ^{32}P

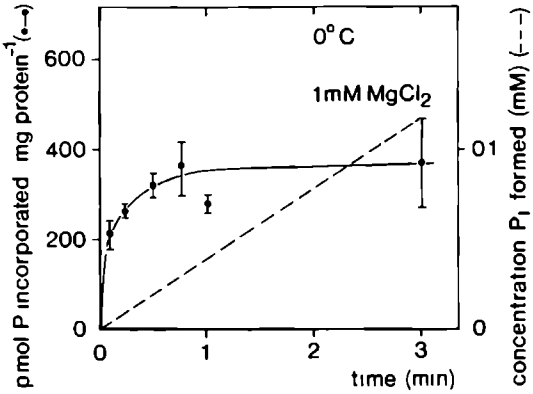


Fig. 4.14 Phosphorylation and enzymatic phosphate release by (^{32}P)-4-nitrophenylphosphate. Phosphorylation of purified Na-K ATPase preparations from rabbit kidney outer medulla by (^{32}P)-NPP is performed at 0°C as described in section 2.8. After various periods of phosphorylation the amount of incorporated P is determined. In a parallel experiment the amount of 4-nitrophenylphosphate hydrolyzed under these conditions is determined as described in section 2.7, and is plotted as P_i formed in mmol/l.

incorporation from (^{32}P) NPP (c. 60 pmol P.mg protein^{-1}.sec^{-1}) is higher than the mean rate of incorporation of $^{32}P_i$ during the first 3 sec of phosphorylation, calculated from fig. 2.4, for a mean P_i concentration of 1 μM, which is derived from the enzymatic hydrolysis curve in fig. 4.14 (0.25 pmol P.mg protein^{-1}.sec^{-1}).This strongly indicates that, at least initially, phosphorylation cannot take place from P_i enzymatically released from (^{32}P) NPP. After 1 min phosphorylation (the time used in most other experiments), sufficient P_i has been released by enzymatic hydrolysis of 4-nitrophenylphosphate to account for the amount of phosphorylation. In view of the monotone phosphorylation curves invariably obtained in our experiments, it is highly unlikely that phosphorylation directly from 4-nitrophenylphosphate is subsequently replaced by phosphorylation from enzymatically released P_i.

The second possibility has been checked by preincubating the enzyme preparation with ouabain and $MgCl_2$ prior to phosphorylation, which precludes enzymatic hydrolysis of the substrate. During phosphorylation

varying concentrations of non-radioactive NPP or inorganic phosphate (imidazole salt) are added to the medium (fig. 4.15). Addition of 2 mM NPP

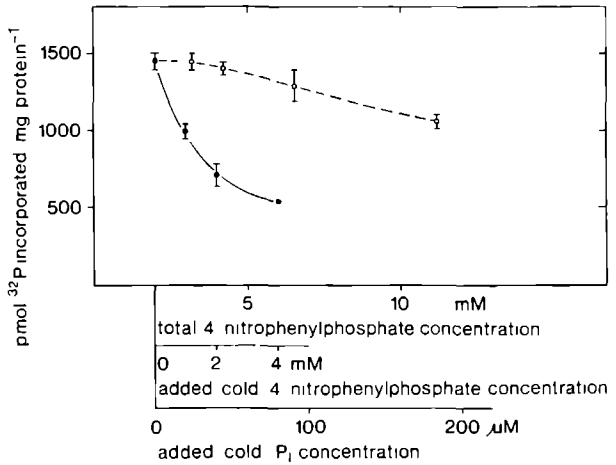


Fig. 4.15 Effects of addition of 4-nitrophenylphosphate and P_i on phosphorylation by (^{32}P) 4-nitrophenylphosphate. Phosphorylation of Na-K ATPase preparations has been carried out after preincubation with 0.12 mM ouabain + 1.4 mM $MgCl_2$, as described in section 2.8. To the phosphorylation medium is added either 'cold' NPP (—●—) or 'cold' P_i (---o---) in the indicated concentrations. Results are corrected for blank phosphorylation.

lowers the amount of phosphorylation by 50%, the same amount by which the specific activity is decreased. Addition of P_i has very little effect, as much as 0.45 mM would be needed in the presence of 2 mM NPP in order to explain the observed phosphorylation. After incubation with 0.45 mM $^{32}P_i$ in the presence of $MgCl_2$ at 0°C, the enzyme should be maximally phosphorylated after 3 min, which is not the case (fig. 4.14). Hence, it is very unlikely that a 23% contamination of the (^{32}P) NPP (which in itself would be an unreasonably large impurity) could explain the phosphorylation by 4-nitrophenylphosphate.

In experiments without ouabain a smaller contamination of $^{32}P_i$ (10 µM) in 2 mM (^{32}P) NPP could in principle account for the phosphorylation. However, the observation that K^+ ions enhance this phosphorylation and not that by P_i (Post et al., 1975) and the relatively short period, after which maximal phosphorylation is reached, confirm the earlier conclusion that the

initial phosphorylation is due to (^{32}P) NPP also in the absence of ouabain.

4.9 Discussion and conclusions

The results presented in this chapter clearly indicate that the SDS extracted microsomes from rabbit kidney Na-K ATPase represent a highly active and highly purified Na-K ATPase preparation with a purity of over 90% on protein basis The preparation still contains most of the phospholipids of the original membranes. Both the partial activities, which appear to represent reactions involving the E_1 enzyme conformation (Na^+ ATPase activity and phosphorylation by ATP), as well as the reactions involving only the E_2 conformation (K^+stimulated 4-nitrophenylphosphatase activity and phosphorylation by P_i) can be determined. This means that no essential changes in overall and partial reaction mechanisms have taken place during purification.

The preparation characterized in this chapter, has proven to be very suitable for chemical modification experiments, which will be described in the next five chapters.

CHAPTER 5

EFFECTS OF N-ETHYLMALEIMIDE ON OVERALL AND PARTIAL ACTIVITIES

5.1 Introduction

N-ethylmaleimide is a reagent, which is generally thought to alkylate sulfhydryl groups at neutral pH (fig. 5.1). It has previously been shown

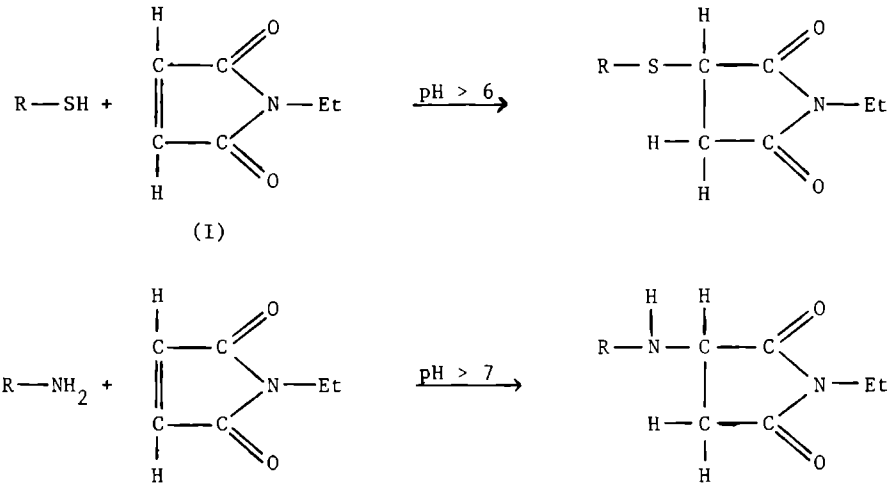


Fig. 5.1 Reaction mechanism of N-ethylmaleimide (I) (Means and Feeney, 1971).

that after reaction with N-ethylmaleimide the Na-K ATPase activity is inhibited (Skou and Hilberg, 1965; Fahn et al., 1966b; Banerjee et al., 1972a and b). The degree of inhibition depends on the kind of ligands (Na^+, K^+, Mg^{2+}, ATP) present during the reaction (Banerjee et al., 1972 a and b).

Conflicting results have been reported about the effects of reaction of N-ethylmaleimide on the overall Na-K ATPase reaction and the partial activities in crude preparations. From a comparison of the effects of

N-ethylmaleimide on the overall reaction and the Na^+-stimulated ADP-ATP phosphate exchange reaction, Fahn et al. (1966b) conclude that N-ethylmaleimide inhibition of the overall reaction occurs through blocking of the transition of an ADP-sensitive phosphorylated intermediate to a K^+-sensitive form (transition $E_1 \sim P \rightarrow E_2 - P$, fig 1.1). On the other hand, there are also arguments against this explanation for the N-ethylmaleimide inhibition mechanism. Klodos and Skou (1975) have raised doubts about the concept of two phosphoenzymes as intermediates in the hydrolysis of ATP by Na-K ATPase. Although Fujita et al. (1966) reported a parallel inhibition by N-ethylmaleimide of the K^+-dependent 4-nitrophenylphosphatase reaction and of the overall reaction, Robinson (1970) finds no inhibition of the former activity. Stimulation of the Na^+-dependent ADP-ATP phosphate exchange activity after inhibition of the Na-K ATPase activity by N-ethylmaleimide has been found in electric eel electroplax microsomes, but not in rat brain microsomes (Fahn et al., 1966b). In contrast to the finding of Siegel et al. (1969), Hegyvary (1976) reports that N-ethylmaleimide does not completely inhibit the ouabain-dependent phosphorylation by P_i. Furthermore, N-ethylmaleimide decreases the number of ATP binding sites without any effect on the affinity of ATP for the residual sites (Nørby and Jensen, 1974).

In view of this conflicting evidence, obtained with crude enzyme preparations, we have investigated the effect of N-ethylmaleimide on a purified preparation. We find a parallel inhibition of all partial reactions and the overall reaction, which appears to rule out the specific blocking of the transition of an ADP-sensitive phosphorylated intermediate to a K^+-sensitive form.

5.2 Materials and methods

Purified Na-K ATPase is prepared from rabbit kidney outer medulla microsomes, as described in section 3.4. The highly active Na-K ATPase preparations thus obtained are incubated for 30 min at 37°C in a medium containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 25 mM imidazole-HCl (pH 7.4) to remove ATP, and are then centrifuged for 10 min at 300.000g. The resulting pellets are washed twice by resuspension and centrifugation in 2 mM CDTA, 25 mM imidazole-HCl (pH 7.5). The preparations are stored at -20°C in a buffer containing 250 mM sucrose, 2 mM CDTA, 25 mM imidazole-

HCl (pH 7.5). They have a specific Na-K ATPase activity of 1000-2000 μmol ATP split.mg protein^{-1}.hr^{-1}, they are free of ouabain-insensitive ATPase activity, and the specific K^+-stimulated, ouabain-inhibited 4-nitrophenylphosphatase activity (K-NPPase) is 200-400 μmole 4-nitrophenylphosphate (NPP) split.mg protein^{-1}.hr^{-1}.

Reaction with N-ethylmaleimide is performed, unless otherwise stated, during 30 min at 37°C in a medium containing 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA, and up to 50 μg protein.ml^{-1}. N-ethylmaleimide and other additives are added in the stated concentrations.

The reaction with N-ethylmaleimide is stopped by addition of 5-fold molar excess of dithioerythritol. Blanks are prepared by adding dithioerythritol to the N-ethylmaleimide solution 10 min prior to addition of the enzyme. After preincubation with N-ethylmaleimide the reaction mixture is kept on ice, and aliquots are taken for determining enzymatic activities.

In some experiments additives, added during the N-ethylmaleimide reaction (e.g. KCl, NPP, ATP), may interfere with the subsequent enzymatic assay. In those cases the preparation is first subjected to column chromatography. The N-ethylmaleimide reaction mixture (100 μl) is placed on a Sephadex G 25 coarse column (100 x 5 mm), equilibrated in 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA. The enzyme is eluted in a 1 ml fraction after 0.9 ml elution with the equilibration buffer.

Although the N-ethylmaleimide solutions are always freshly prepared, there is some variation in the degree of inhibition, apparently due to slow decomposition of N-ethylmaleimide in the solid state. The inhibition curve in fig. 5.5 has, therefore, been determined with a fresh batch of N-ethylmaleimide.

Enzyme activities are determined as described in chapter 2.

Ouabain preincubation has been performed as described in section 4.5.

Materials. ADP (free acid) and adenylylimidodiphosphate (Li_4 salt) are supplied by Boehringer (Mannheim, W-Germany). All other chemicals are of reagent grade.

5.3 Results

5.3.1 Effects of N-ethylmaleimide on the Na-K ATPase activity

Preincubation of purified Na-K ATPase with N-ethylmaleimide causes inactivation of the ATPase activity. Fig. 5.2 shows that the inhibition

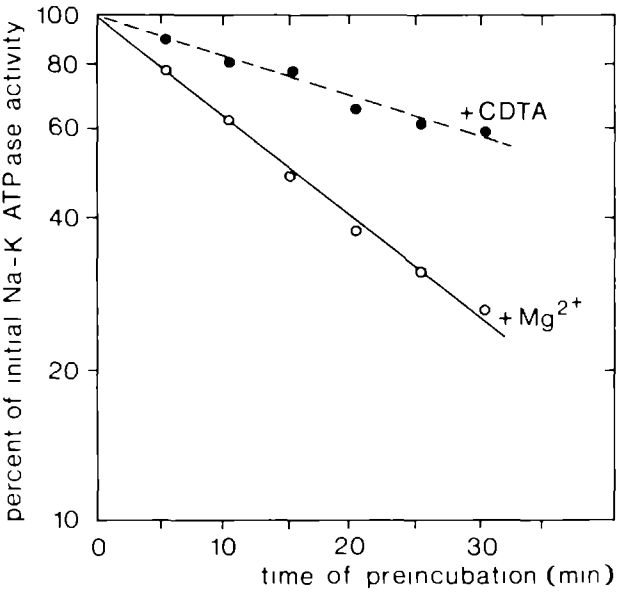


Fig. 5.2 Inhibition of Na-K ATPase activity by N-ethylmaleimide. The reaction mixture for preincubation with N-ethylmaleimide contains: 7.5 µg protein.ml^{-1}, 2 mM CDTA (•) or 5 mM $MgCl_2$ (o), 0.7 mM N-ethylmaleimide, 25 mM imidazole-HCl (pH 7.5). After varying times of preincubation at 37°C, aliquots are removed for the assay of Na-K ATPase activity as described in section 2.3 (method I).

rate follows pseudo first order kinetics, both in the presence and absence of Mg^{2+} ions. In the presence of Mg^{2+} ions the inhibition rate is higher. In all further experiments the reaction with N-ethylmaleimide is performed in the absence of Mg^{2+} ions and in the presence of CDTA, except when cation effects are studied (section 5.3.3).

Addition of ATP, ADP or adenylylimidodiphosphate to the N-ethylmaleimide reaction mixture (10 mM N-ethylmaleimide, 30 min preincubation) lowers the inhibition of the Na-K ATPase activity. Their half-maximal concentrations are 35, 39 and 160 µM respectively (fig 5.3). ATP protects only when it is bound to the enzyme under non-phosphorylating conditions, i.e. in the absence of Mg^{2+}. Addition of $MgCl_2$ during the reaction with N-ethylmaleimide under these conditions abolishes the protective effect (Table 5.1). Partial protection (up to 25%) against inhibition of the Na-K ATPase activity by N-ethylmaleimide is given by 4-nitrophenylphosphate.

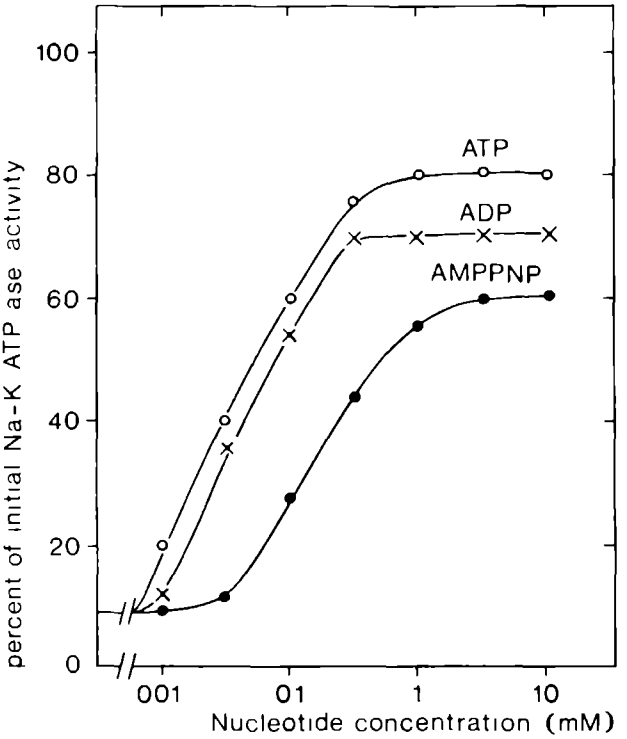


Fig. 5.3 Effect of nucleotides on the inhibition of Na-K ATPase activity by N-ethylmaleimide. The reaction with N-ethylmaleimide is carried out at 37°C for 30 min in media containing 7.5 µg protein.ml^{-1}, 10 mM N-ethylmaleimide, 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA (5 mM when ATP is present) and ATP (Tris salt) (o) or ADP (imidazole salt) (×) or adenylylimidophosphate (tetra-lithium salt; AMPPNP) (•) in the concentrations indicated. After reaction with N-ethylmaleimide the Na-K ATPase activity is determined as described in section 2.3 (method I). Corrections are made for the effects of added nucleotides on the Na-K ATPase activity.

This effect also seems to depend on mere binding of 4-nitrophenylphosphate to the enzyme, since it disappears when phosphorylation of the enzyme by this substance (section 4.8) can occur (presence of Mg^{2+} with or without K^+, Table 5.1).

The inhibitory effect of N-ethylmaleimide is pH-dependent, increasing with increasing pH (fig. 5.4). The persistence of inhibition at pH values below 7.0 indicates reaction with sulfhydryl groups (Means and Feeney, 1971). In all further experiments the reaction with N-ethylmaleimide has

Table 5.1

EFFECT OF VARIOUS LIGANDS ON INHIBITION BY N-ETHYLMALEIMIDE

Ligands added during N-ethylmaleimide reaction	Remaining activity (%)	
	K-NPPase	Na-K ATPase
Control (no N-ethylmaleimide)	100	100
4 mM CDTA	7.6 ± 0.6	6.2 ± 0.4
4 mM CDTA + 10 mM ATP	56.8 ± 2	69.5 ± 7
10 mM ATP + 100 mM NaCl + 5 mM $MgCl_2$	14.4 ± 3.5	7.6 ± 1.8
4 mM CDTA + 10 mM 4-nitrophenylphosphate	26.1 ± 1.8	21.1 ± 2
10 mM 4-nitrophenylphosphate + 5 mM $MgCl_2$	13.7 ± 0.9	5.5 ± 0.1
10 mM 4-nitrophenylphosphate + 5 mM $MgCl_2$ + 5 mM KCl	6.8 ± 1.2	4.6 ± 1.3

Treatment with N-ethylmaleimide is carried out in a medium containing. 10 mM N-ethylmaleimide (except for the control), 25 mM imidazole-HCl (pH 7.5), 100 µg protein.ml^{-1} and ligands as indicated in the table, during 30 min at 37°C. After reaction with N-ethylmaleimide the ligands are removed by gel filtration on a Sephadex G 25 column, and assays for K^+ stimulated 4-nitrophenylphosphatase (K-NPPase) and Na-K ATPase activity are performed as described in sections 2.3 (method I) and 2.7. Results of 3 experiments carried out in duplicate are presented as means with standard error of the mean.

taken place at pH 7.5.

Inhibition as a function of the N-ethylmaleimide concentration during preincubation at pH 7.5 is shown in fig. 5.5. Half-maximal inhibition is obtained at 0.7 mM (pI_{50} = 3.2).

5.3.2 Effects of N-ethylmaleimide on the partial reactions of the Na-K ATPase activity

Preincubation of Na-K ATPase preparations with N-ethylmaleimide leads to parallel reduction of the Na-K ATPase and K-NPPase activities, independent of the pH of the reaction mixture (fig. 5.4), and of the concentration of N-ethylmaleimide (fig. 5.5).

The degree of inhibition of the K-NPPase activity by preincubation with various concentrations of N-ethylmaleimide is independent of the kind and concentrations of ligands present during assay (fig. 5.6). The stimulation of this activity, in the presence of 0.5 mM K^+, by 20 mM Na^+

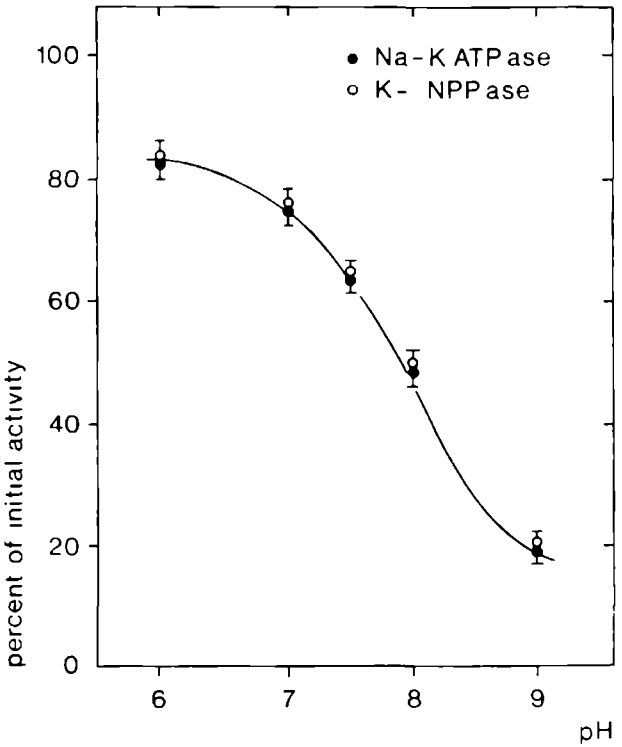


Fig. 5.4 Effect of pH on N-ethylmaleimide inhibition. Na-K ATPase (•) and K^+-stimulated 4-nitrophenylphosphatase (K-NPPase) (o) activities are assayed in aliquots of reaction mixtures containing: 10 µg protein.ml^{-1}, 0.7 mM N-ethylmaleimide, 2 mM CDTA and 50 mM tris-maleate buffer at the indicated pH values. After preincubation for 30 min at 37°C the N-ethylmaleimide reaction is stopped by adding dithio-erythritol to a final concentration of 10 mM. Corrections are made for the spontaneous inactivation of the enzyme at the given pH.

(20% stimulation) or by 20 mM Na^+ + 0.1 mM ATP (150% stimulation) is not changed by reaction with N-ethylmaleimide.

As shown in table 5.2, the Na^+ stimulated ATPase activity is inhibited to the same extent as the Na-K ATPase activity. Moreover, the inhibition of the Na^+ stimulated ATPase activity by K^+ ions remains after N-ethylmaleimide treatment, suggesting that the affinity of K^+ ions for the inhibitory site is not changed.

Na-K ATPase preparations can be phosphorylated by ATP in the presence of Mg^{2+} and Na^+ ions. As fig. 5.7 shows, the degree of phosphorylation and

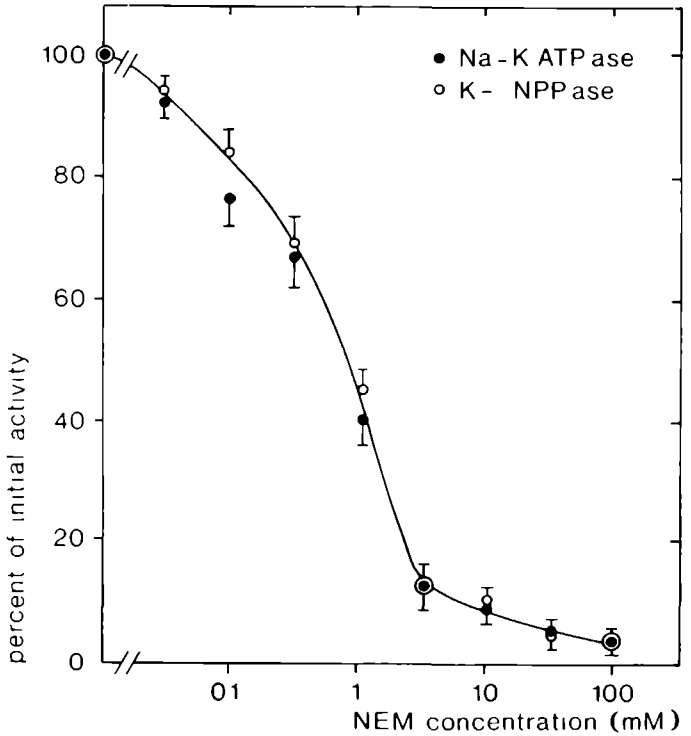


Fig. 5.5 N-ethylmaleimide inhibition of Na-K ATPase and K^+-stimulated 4-nitrophenylphosphatase activities as a function of reagent concentration. The N-ethylmaleimide reaction mixture contains. 7.5 µg protein.ml^{-1}, 2 mM CDTA, 25 mM imidazole-HCl (pH 7.5) and N-ethylmaleimide (NEM) at stated concentrations. After preincubation for 30 min at 37°C the N-ethylmaleimide reaction is ended by adding dithio-erythritol (5-fold molar excess), and aliquots of the mixture are assayed for Na-K ATPase activity (•) and K^+-stimulated 4-nitrophenylphosphatase (K-NPPase) activity (o). Fresh batches of N-ethylmaleimide have been used in these experiments.

the Na-K ATPase activity are decreased to the same extent after preincubation with various concentrations of N-ethylmaleimide. This is true, whether the phosphorylation is performed at 0°C (fig. 5.7) or at 37°C (results not shown).

The enzyme can also be phosphorylated by P_i. In previous studies with crude enzyme preparations, preincubation with ouabain and Mg^{2+} ions was necessary to obtain appreciable phosphorylation. In our highly purified preparations phosphorylation by P_i in the absence of ouabain is not much less than that in its presence (section 4.5). This phosphorylation

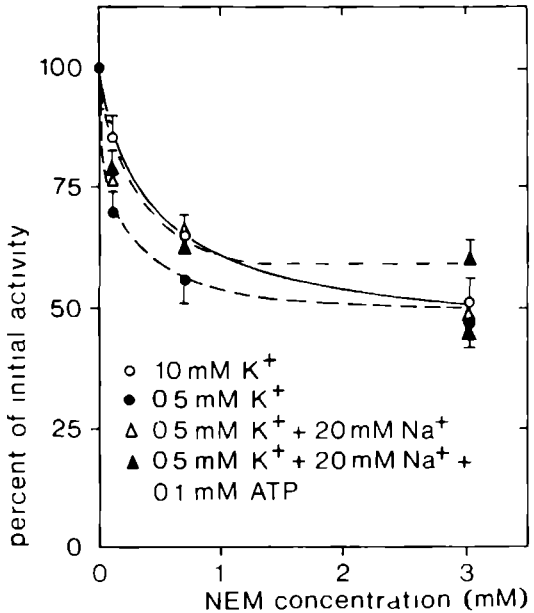


Fig. 5.6 Influence of N-ethylmaleimide on K^+ stimulated 4-nitrophenylphosphatase activity in the presence of various ligands. Preincubation with N-ethylmaleimide (NEM) is carried out for 30 min at 37°C in media containing 10 µg protein.ml^{-1}, 2 mM CDTA, 25 mM imidazole-HCl (pH 7.5) and N-ethylmaleimide at the indicated concentrations. The reaction is ended by adding a 5-fold molar excess of dithiothreitol. K^+-stimulated 4-nitrophenylphosphatase (K-NPPase) activity is determined as described in section 2.7, in the presence of 10 mM KCl (o) or 0.5 mM KCl (•) or 0.5 mM KCl + 20 mM NaCl (Δ) or 0.5 mM KCl + 20 mM NaCl + 0.1 mM ATP (▲). The K-NPPase activities are presented as percent of initial activity.

Table 5.2

EFFECTS OF N-ETHYLMALEIMIDE ON Na^+-STIMULATED ATPase ACTIVITY

Concentration of N-ethylmaleimide during preincubation	Na^+ stim. ATPase*		Na-K ATPase*
	no KCl	10 mM KCl	
0 mM	100	55 ± 1	= 100
2 mM	32 ± 2	13 ± 4	33 ± 3

*percent of initial activity. The preincubation of the enzyme is carried out in medium containing. 2 mM CDTA, 25 mM imidazole-HCl (pH 7.5), 10 µg protein .ml^{-1} and N-ethylmaleimide at a concentration as indicated, during 30 min at 37°C. After stopping the preincubation by addition of dithioerythritol to a final concentration of 10 mM, aliquots are assayed for Na^+-stimulated ATPase and Na-K ATPase activity as described in sections 2.4 and 2.3 (method II). Results of 2 experiments carried out in duplicate are presented as means with standard error of the mean.

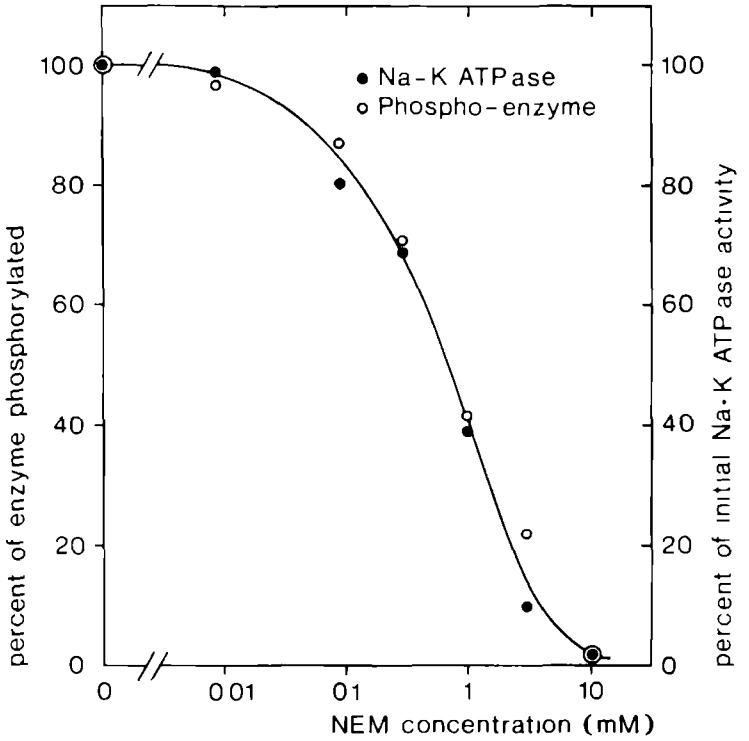


Fig. 5.7 N-ethylmaleimide inhibition of phosphorylation by ATP and of Na-K ATPase activity. Preincubation with N-ethylmaleimide (NEM) is carried out for 30 min at 37°C in a medium containing 200 µg protein.ml^{-1}, 2 mM CDTA, 25 mM imidazole-HCl (pH 7.5) and N-ethylmaleimide at the indicated concentrations. After stopping the reaction, the phosphorylation by ATP at 0°C (o) and Na-K ATPase activity (●) are determined as described in sections 2.5 and 2.3 (method I).

reaction is also inhibited by prior reaction with N-ethylmaleimide, and again to about the same extent as the Na-K ATPase activity. Inhibition of the two activities as a function of the N-ethylmaleimide concentration is shown in fig. 5.8. In other experiments the phosphorylation has been determined as a function of the P_i concentration. From these data the dissociation constant of the phosphate-enzyme complex has been calculated by means of a Scatchard plot. It appears that the dissociation constant is not noticeably changed by prior treatment with N-ethylmaleimide, but that the number of binding sites is reduced (table 5.3).

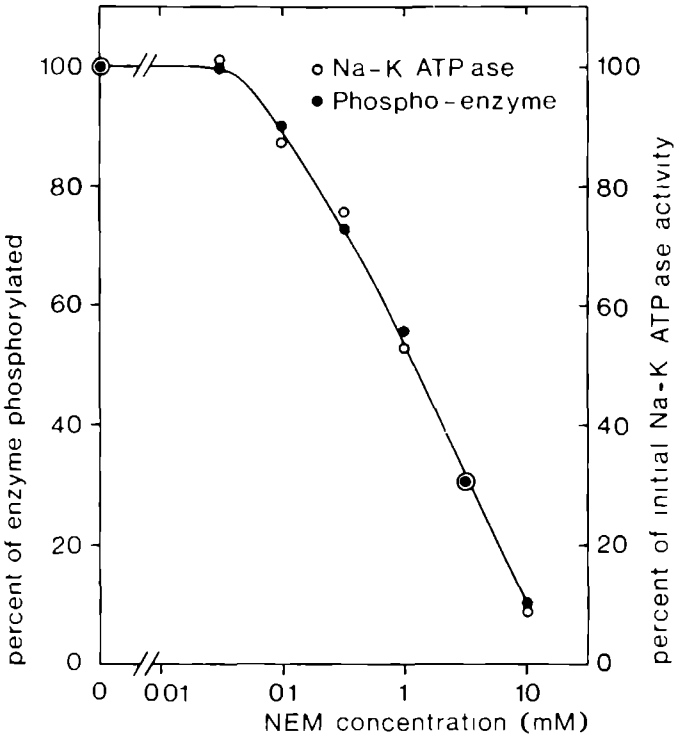


Fig. 5.8 N-ethylmaleimide inhibition of phosphorylation by P_i in the presence of ouabain and of Na-K ATPase activity. Preincubation with N-ethylmaleimide (NEM) is carried out as described in the legend of fig. 5.7 Thereafter aliquots are taken for ouabain treatment and subsequent phosphorylation by 50 μM P_i (•) and for Na-K ATPase assay (o), as described in sections 2.6 and 2.3 (method I).

5.3.3 Effects of Na^+, K^+ and Mg^{2+} ions on inhibition by N-ethylmaleimide

Addition of Na^+, K^+ or Mg^{2+} ions to the N-ethylmaleimide reaction mixture influences the rate of Na-K ATPase inhibition. In fig. 5.9 a and b the effects of these ions on the inhibition are shown. Choline chloride is added in order todetermine ionic strength effects. No significant ionic strength effect on the N-ethylmaleimide inhibition is found. Na^+ and Mg^{2+} ions both increase the inhibition, when included in the N-ethylmaleimide reaction mixture. For both ions the effect is maximal at 10 mM concentration. At higher ion concentrations there is less inhibition; with Na^+ ions in concentrations over 50 mM the increase even disappears. Addition of up

Table 5.3

EFFECT OF N-ETHYLMALEIMIDE TREATMENT ON PHOSPHORYLATION BY INORGANIC PHOSPHATE

	with ouabain treatment	without ouabain treatment
Na-K ATPase activity, remaining after NEM treatment	45%	72%
Fraction of P_i binding sites remaining after NEM treatment	60%	73%
K_{diss} of the enzyme-P_i complex,		
a. without NEM treatment	21 µM	36 µM
b. after NEM treatment	18 µM	31 µM

Treatment with N-ethylmaleimide (NEM) is carried out in a medium containing 0.7 mM N-ethylmaleimide, 25 mM imidazole-HCl (pH 7.5), 200 µg protein.ml^{-1}, 2 mM CDTA, during 30 min at 37°C. The N-ethylmaleimide reaction is terminated by addition of excess dithioerythritol. Ouabain treatment is carried out as described in section 4.5. After treatment with N-ethylmaleimide Na-K ATPase activity and phosphorylation capacity at varying P_i concentrations (1-100 µM) are determined as described in sections 2.6 and 2.3 (method I).

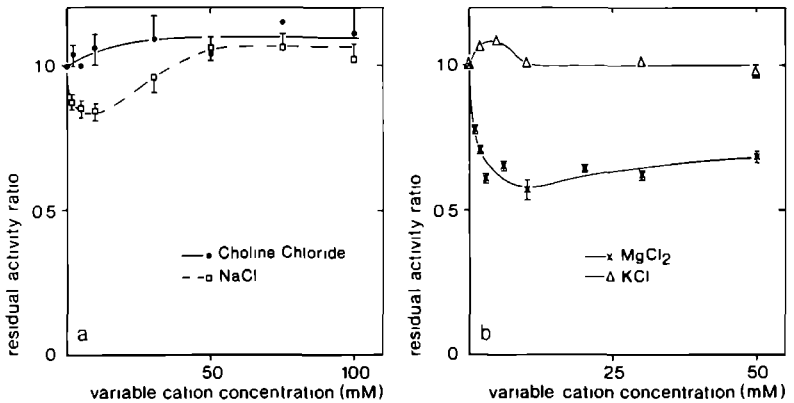


Fig. 5.9 a and b Effects of Na^+, K^+, Mg^{2+} and $Choline^+$ ions on inhibition of Na-K ATPase activity by N-ethylmaleimide. The N-ethylmaleimide (NEM) reaction mixture contains: 0.3 mM NEM, 2 mM CDTA (omitted when $MgCl_2$ is added, 25 mM imidazole-HCl (pH 7.5), 7.5 µg protein.ml^{-1} and NaCl (□), KCl (Δ), $MgCl_2$ (×) or Choline chloride (●) at stated concentrations. After preincubation during 30 min at 37°C the NEM reaction is ended by addition of a 5-fold molar excess of dithioerythritol and aliquots of the mixture are assayed for Na-K ATPase activity as described in section 2.3 (method I). The ratio of the residual activities is plotted. At the ratio 1.0 the residual activity is 70%.

to 10 mM KCl to the N-ethylmaleimide reaction mixture results in a reduced inhibition, while at higher concentrations the effect disappears.

The effects of addition of combinations of cations on the N-ethylmaleimide inhibition have also been studied. The addition of Na^+ or K^+ ions to the N-ethylmaleimide inhibition mixture in the presence of a fixed 10 mM $MgCl_2$ concentration causes only a small decrease in inhibition (fig. 5.10a).

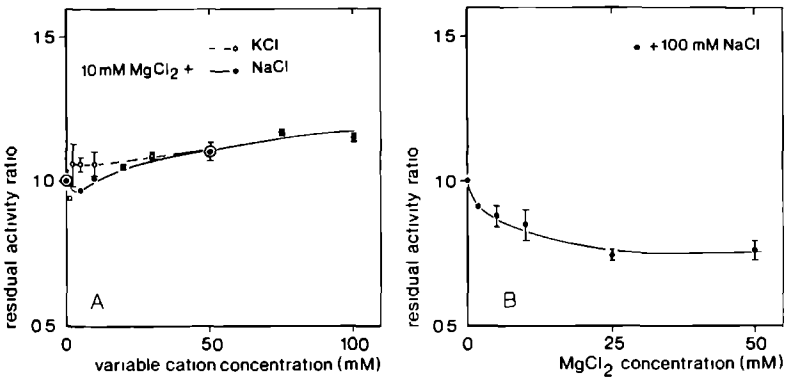


Fig. 5.10 a and b Effects of combinations of cations on inhibition of Na-K ATPase activity by N-ethylmaleimide.
a. The N-ethylmaleimide (NEM) reaction mixture contains: 0.3 mM NEM, 10 mM $MgCl_2$, 25 mM imidazole-HCl (pH 7.5), 7.5 µg protein.ml^{-1} and either NaCl (—•—) or KCl (- -o-·-) at the stated concentrations. After preincubation during 30 min at 37°C the NEM reaction is terminated by addition of a 5-fold molar excess of dithioerythritol, and aliquots of the mixture are assayed for Na-K ATPase activity as described in section 2.3 (method I). The ratio of residual activities is plotted. At the ratio 1.0 the residual activity is 70%.
b. The NEM reaction mixture contains: 0.3 mM NEM, 100 mM NaCl, 25 mM imidazole-HCl (pH 7.5), 7.5 µg protein.ml^{-1} and $MgCl_2$ at the stated concentrations. Preincubation and residual activity determinations are performed as described under a. At the ratio 1.0 the residual activity is 70%.

These effects are completely different from those of addition of Na^+ or K^+ ions alone to the N-ethylmaleimide reaction mixture (cf. fig. 5.9 a and b). Addition of increasing concentrations of $MgCl_2$ to the N-ethylmaleimide reaction mixture in the presence of a fixed 100 mM NaCl concentration results in an increased inhibition (fig. 5.10b). However, in this case the maximally effective concentration of Mg^{2+} is higher than that in the

case of addition of Mg^{2+} alone (25 mM vs. 10 mM, cf. fig. 5.9b).

5.3.4 Effects of ADP and inorganic phosphate on inhibition by N-ethylmaleimide

Addition of ATP (under non-phosphorylating conditions) will induce the E_1 conformation of the enzyme (see chapter 1, fig. 1). The same is true for ADP, which cannot phosphorylate, but binds with about the same affinity as ATP (Nørby and Jensen, 1971). When ADP is added to the N-ethylmaleimide reaction mixture at a concentration that will saturate the high affinity site, no effect of addition of NaCl on N-ethylmaleimide inhibition can be observed (fig. 5.11). Addition of P_i in concentrations above 0.1 mM,

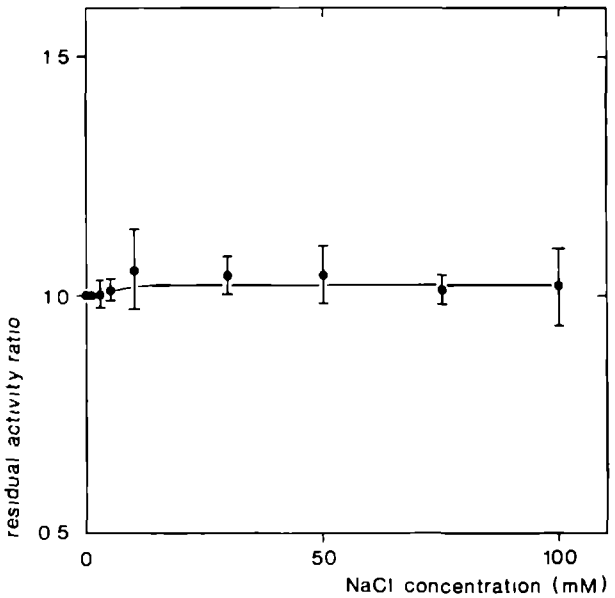


Fig. 5.11 Effects of ADP and NaCl on the inhibition of Na-K ATPase by N-ethylmaleimide. The N-ethylmaleimide (NEM) reaction mixture contains: 0.3 mM NEM, 10 µM ADP, 25 mM imidazole-HCl (pH 7.5), 7.5 µg protein.ml^{-1}, and NaCl at the stated concentrations. After preincubation during 30 min at 37°C the NEM reaction is stopped by addition of a 5-fold molar excess of DTE and aliquots of the mixture are assayed for Na-K ATPase activity as described in section 2.3 (method I). The ratio of the residual activities is plotted. At the ratio 1.0 the residual activity is 75%.

combined with 10 mM $MgCl_2$, reduces the N-ethylmaleimide inhibition (fig. 5.12).

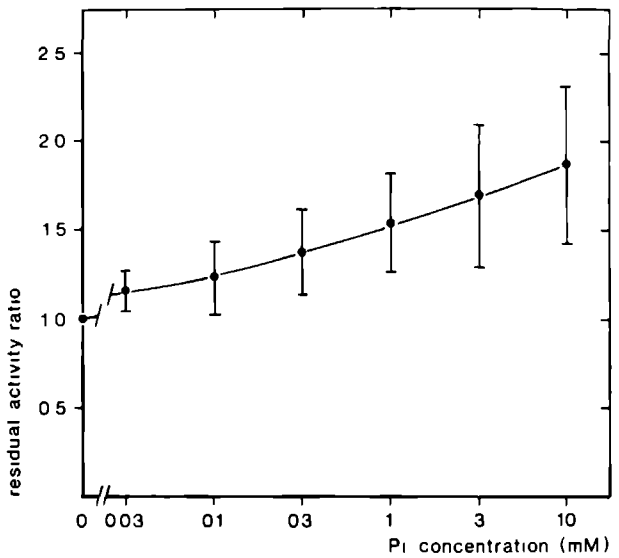


Fig. 5.12 Effects of inorganic phosphate on the inhibition of Na-K ATPase activity by N-ethylmaleimide. The N-ethylmaleimide (NEM) reaction mixture contains: 0.3 mM NEM, 10 mM $MgCl_2$, 25 mM imidazole-HCl (pH 7.5), 7.5 µg protein.ml^{-1} and imidazole phosphate at the stated concentrations. After preincubation during 30 min at 37°C the NEM reaction is stopped by addition of a 5-fold molar excess of DTE, and aliquots of the mixture are assayed for Na-K ATPase activity as described in section 2.3 (method I). The ratio of the residual activities is plotted. At the ratio 1.0 the residual activity is 30%.

5.4 Discussion

The experiments reported in this chapter clearly indicate that the purified Na-K ATPase from rabbit kidney outer medulla is inhibited by pre-treatment with N-ethylmaleimide, and that this inhibition is antagonized by nucleotides and 4-nitrophenylphosphate under conditions excluding phosphorylation. Our study further shows that the partial reactions are also inhibited. Their inhibition is in all cases equal to that of the overall activity.

The latter finding is in contrast with several previous reports. Our

results confirm the parallel inhibition of Na-K ATPase and K-NPPase activity observed by Fujita et al. (1966). The main discrepancy between our results and those previously reported concerns the equal inhibition by N-ethylmaleimide of Na-K ATPase activity and of phosphorylation by ATP (Fahn et al., 1966b, 1968, Post et al., 1965) and by P_i (Hegyvary, 1976, Siegel et al., 1969). Two possible explanations for this discrepancy occur to us. First, in nearly all previous studies crude enzyme preparations have been used. This means that phosphorylation experiments may have given appreciable errors due to phosphorylation of contaminating proteins. Secondly, the purified preparation may differ from the crude preparations with respect to accessibility of groups reacting with N-ethylmaleimide. As previously suggested by Banerjee et al. (1972a), and confirmed by our experiments described in chapter 7, more than one group reacts with N-ethylmaleimide with concomitant loss of enzyme activity. Exposure of these groups may depend on the conformational state of the enzyme. Since the conformational state may depend on the presence of certain cations and other ligands, we have washed our preparations in order to remove Na^+, K^+, Mg^{2+} and ATP.

There is some uncertainty about the nature of the group, which reacts with N-ethylmaleimide. Previous authors, reporting inhibition of Na-K ATPase activity by treatment with N-ethylmaleimide, have always assumed that reaction with a sulfhydryl group is responsible. Our finding that inhibition of Na-K ATPase also occurs at pH values below 7, may indicate that N-ethylmaleimide does react with sulfhydryl groups, since reaction with amino groups will mainly take place at pH values above 8.0 (Means and Feeney, 1971). However, reaction of N-ethylmaleimide with amino acid or protein amino groups can occur at a pH as low as 7.0 (Smyth et al., 1960, 1964; Guidotti and Konigsberg, 1964). Since however inhibition of Na-K ATPase activity through modification of amino groups by means of alkylimidate compounds is not antagonized by ATP (de Pont et al., to be published), there is good reason to believe that in our experiments the reaction has been with sulfhydryl groups.

The conclusion of Fahn et al. (1966b) that the main effect of N-ethylmaleimide would be the inhibition of the conversion of an ADP-sensitive ($E_1 \sim P$) to a K^+-sensitive phosphorylated intermediate (E_2-P) is not supported by our results. Their conclusion conflicts with the equal

inhibition of the overall Na-K ATPase reaction and of the binding of ATP (Nørby and Jensen, 1974), of the phosphorylation by ATP and of the Na^+-activated ATPase activity, all of which represent reactions preceding the formation of an E_1-P intermediate.

Our results lead us to a different explanation for the effects of N-ethylmaleimide. We propose that this substance reacts progressively with one or more vital functional groups (most likely sulfhydryl groups) of the enzyme, thereby completely abolishing the activity of an increasing number of enzyme molecules. Support for this assumption is derived from: 1. the equal inhibition of partial reactions and overall reaction of the enzyme system, 2. the lack of change in the parameters of the remaining enzyme activity, viz. the dissociation constants for the enzyme-ATP complex (Nørby and Jensen, 1974) and for the enzyme-P_i complex, and the stimulation of the K-NPPase activity (at 0.5 mM K^+) by 20 mM Na^+ with or without 0.1 mM ATP.

This vital functional group could be located either inside or outside the catalytic center of the enzyme system. The first possibility is favored by the protective effects of ATP and its analogues and of 4-nitrophenylphosphate, and also by the finding that phosphorylation by ATP increases the number of moles N-ethylmaleimide bound per mole Na-K ATPase (Hart and Titus, 1973). On the other hand, substances which appear to react with a group inside the catalytic center, like sulfhydryl reactive ATP analogues (Patzelt-Wenczler et al., 1975) and 2,3-butanedione (chapter 8) inhibit K-NPPase activity less than the Na-K ATPase activity, whereas N-ethylmaleimide inhibits these two activities equally.

Favoring a location of the vital functional group outside the catalytic center is the following consideration. Evidence has accumulated that the Na-K ATPase molecule has two catalytic centers with different affinities (Robinson, 1976; Glynn and Karlish, 1976). The center with high affinity is thought to be responsible for the Na^+-stimulated ATPase activity, while the low affinity center would catalyse the K-NPPase reaction, the overall reaction involving both centers (Robinson, 1976). This model rules out equal inhibition of partial and overall reactions upon reaction with a vital functional group in only one of the catalytic centers. More likely, reaction with such a group outside the catalytic centers could affect both catalytic centers equally by modification of the interaction of the sub-

units. The parallel protective effects of nucleotides and 4-nitrophenylphosphate resemble that of ATP against inactivation by sodium dodecylsulfate (fig. 2 in Jørgensen, 1974a), either by stabilization of the active enzyme molecule or by an induced conformational change (Skou, 1974).

Banerjee et al. (1972 a and b) have already reported effects of cations on the inhibition by N-ethylmaleimide. They differentiate between effects on several partial reactions of Na-K ATPase due to addition of certain cations at fixed concentrations to the N-ethylmaleimide inhibition mixture However, we observe that the effects of cations added to the N-ethylmaleimide inhibition mixture depend on their concentrations. We may assume that these cation effects are due to changes in the enzyme conformation. If not, it would be difficult to explain why most cation effects on the N-ethylmaleimide inhibition disappear at high cation concentrations (cf. fig. 5 8 a and b). A change in enzyme conformation will change the exposure of essential groups. At least in the case of addition of Mg^{2+} ions, no change in pseudo-first order inhibition kinetics occurs (fig. 5.2).

Induction of conformational changes of Na-K ATPase by addition of Na^+ or K^+ ions has been observed by Jørgensen (1975c) during tryptic digestion of the enzyme. A change in enzyme conformation due to addition of Mg^{2+} ions is suggested after calorimetric measurements by Kuriki et al. (1976), but these effects are not completely clear. Jørgensen (1975c) shows that addition of either 150 mM NaCl or 150 mM KCl to the tryptic digestion medium results in different breakdown patterns, due to different enzyme conformations. At such high concentrations, neither NaCl nor KCl seems to affect N-ethylmaleimide inhibition

Because of the biphasic effects of addition of Na^+, K^+ and Mg^{2+} ions on inhibition of Na-K ATPase activity by N-ethylmaleimide we may assume that binding sites with different affinities are involved in the binding of these ions. The non-additive effects of Na^+ or K^+ ions on inhibition by N-ethylmaleimide in the presence of Mg^{2+} ions may be explained by interaction between binding of Mg^{2+} and either Na^+ or K^+ ions to the enzyme.

After addition of ADP, no effect of addition of Na^+ ions on inhibition by N-ethylmaleimide is observed (fig. 5.11). At this concentration of ADP (10 μM), the enzyme will be in the E_1 conformation. The lack of effects by addition of Na^+ ions confirms this, since addition of Na^+ ions also tends to convert the enzyme into an E_1 conformation. The enzyme can be

phosphorylated by P_i in the presence of Mg^{2+} ions. The dissociation constant for the enzyme-P_i complex is 34 μM so at 100 μM P_i the phosphorylation level will exceed 70% of the maximal level. The first effects of P_i on N-ethylmaleimide inhibition are observed at concentrations over 100 μM. This suggests that these effects are due to complexation of Mg^{2+} ions rather than to changes in phosphorylation per se. A decrease in free Mg^{2+} ion concentration below 10 mM decreases the N-ethylmaleimide inhibition (cf. fig. 5.9b). In conclusion, we may say that changes in enzyme conformation may result in different degrees of inhibition.

The equal inhibition of partial reactions and overall reaction of the Na-K ATPase system seriously limits the usefulness of N-ethylmaleimide for the elucidation of the reaction mechanism of this enzyme system. However, the finding that the inhibition by N-ethylmaleimide is influenced by the presence of ligands may be useful in studies of the various conformational states of the enzyme.

CHAPTER 6

EFFECTS OF 5,5' DITHIOBIS (2-NITROBENZOIC ACID) ON THE OVERALL AND PARTIAL REACTIONS

6.1 Introduction

In the preceding chapter we have shown that Na-K ATPase activity can be inhibited by the reaction of N-ethylmaleimide with one or more sulfhydryl groups of the enzyme. We have found no indication for a reaction of N-ethylmaleimide with an essential sulfhydrylgroup inside the ATP binding center (Patzelt-Wenczler et al., 1975). In this chapter we shall show the effects of treatment of Na-K ATPase with another sulfhydryl reagent, 5,5' dithiobis (2-nitrobenzoic acid). The reaction scheme is given in fig. 6.1. It is specific for sulfhydryl groups (Ellman, 1959). The effects

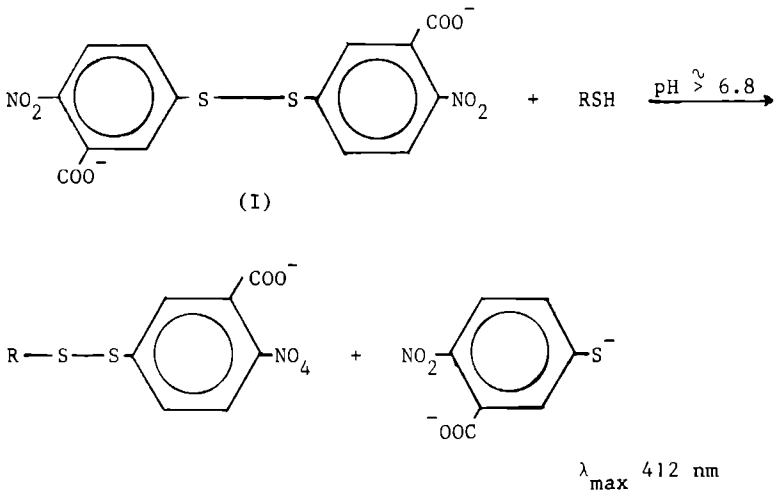


Fig. 6.1 Reaction between protein sulfhydryl groups and 5,5' dithiobis (2-nitrobenzoic acid) (I).

of treatment with 5,5' dithiobis (2-nitrobenzoic acid) on the overall and partial reaction of the enzyme system will be compared with those of

N-ethylmaleimide. The study of cation effects on inhibition of Na-K ATPase activity by reaction with 5,5' dithiobis (2-nitrobenzoic acid) should enable us to differentiate between different enzyme conformations. The observed differences in inhibitory effects of the two compounds lead us to the conclusion that different essential groups are involved in their actions.

6.2 Methods

Purified Na-K ATPase is obtained from microsomes of rabbit kidney outer medulla by extraction with SDS and continuous sucrose density gradient centrifugation as described in section 3.4. The preparation is freed from ATP and washed as described in section 5.2. Na-K ATPase activity and partial reactions are determined as described in chapter 2.

Incubation with 5,5' dithiobis (2-nitrobenzoic acid) (obtained from BDH, Poole, England) is generally performed at 37°C in a medium containing 25 mM imidazole-HCl, 2 mM CDTA, 100 µg protein.ml^{-1} and 5,5' dithiobis (2-nitrobenzoic acid) at the stated concentrations, at pH 7.5 for 60 min. The reaction is stopped by 30-fold dilution with 25 mM imidazole-HCl, 2 mM CDTA (pH 7.5). The diluted reaction mixture is kept on ice, and aliquots are used within minutes for assay of enzyme activities.

When additives present during preincubation with 5,5' dithiobis (2-nitrobenzoic acid), like KCl, NPP and ATP, may interfere with the subsequent assay, the preparation is first subjected to column chromatography. The reaction mixture (100 µl) is placed on a column (Sephadex G25 coarse, 100 x 5 mm) equilibrated in 25 mM imidazole-HCl, 2 mM CDTA (pH 7.5). The enzyme is eluted in a 1000 µl fraction, free of contaminants, after 900 µl elution with equilibration buffer.

6.3 Results

6.3.1 Effect on Na-K ATPase activity

Preincubation of purified Na-K ATPase with 5,5' dithiobis (2-nitrobenzoic acid) causes inactivation of the enzyme activity. The inhibition follows pseudo first order kinetics (fig. 6.2a), as is also the case for inhibition by N-ethylmaleimide (fig. 5.2). From the slope of a double logarithmic plot of the time for 50% inactivation against the inhibitor

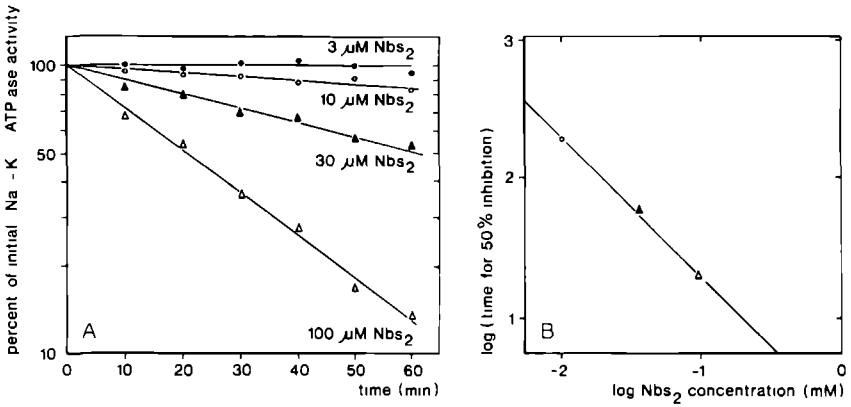


Fig. 6.2 A. Time course for the inhibition of Na-K ATPase activity by 5,5' dithiobis (2-nitrobenzoic acid). The reaction mixture for treatment with 5,5' dithiobis (2-nitrobenzoic acid) (abbreviated: Nbs_2) contains 100 μg protein.ml^{-1}, 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA and various concentrations of 5,5' dithiobis (2-nitrobenzoic acid) (● 3.10^{-3} mM; o 10^{-2} mM; ▲ 3.10^{-2} mM; Δ 10^{-1} mM). After incubation (at 37°C) the reaction mixture is diluted 30-fold, and assayed for Na-K ATPase activity as described in section 2.3 (method I).
B. Double logarithmic plot of time for 50% inhibition vs. inhibitor concentration. Meaning of symbols as in fig. 6.2A.

concentration, the number of molecules 5,5' dithiobis (2-nitrobenzoic acid) reaction per enzyme molecule can be calculated (Keech and Farrant, 1968) A value of 1.0 is found, which indicates true second order kinetics for the reaction (fig. 6.2B), as is also the case for N-ethylmaleimide.

When preincubation is carried out for 60 min at 37°C in a medium containing 100 μg protein.ml^{-1}, 2 mM CDTA, 25 mM imidazole-HCl (pH 7.5), the enzyme is completely inhibited by 1 mM 5,5' dithiobis (2-nitrobenzoic acid), and is 50% inhibited by 20-50 μM of this reagent (fig. 6.3). The rate of inhibition increases with a rise in pH (fig. 6.4). Addition of ATP to the preincubation medium antagonizes the inhibitory effect of 5,5'-dithiobis (2-nitrobenzoic acid) (fig. 6.5), but only under conditions where no phosphorylation can occur, i.e. in the absence of Mg^{2+} and Na^{+} ions (Table 6.1). Half maximal protection is obtained with 30 μM ATP (fig. 6.5). Addition of NPP has no effect on the inhibition.

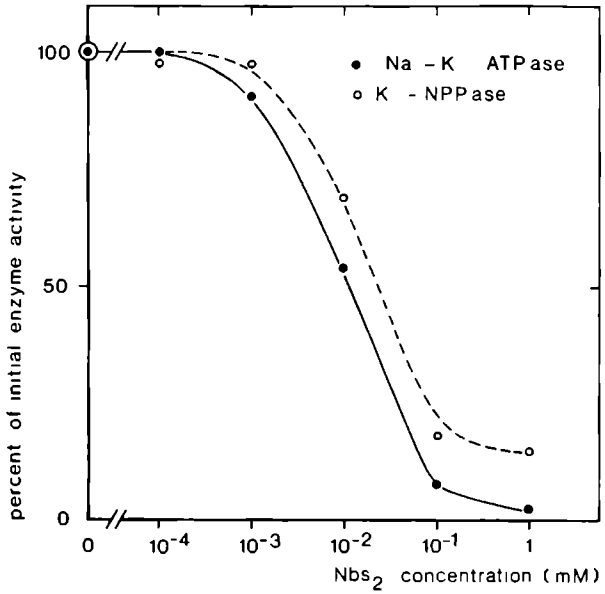


Fig. 6.3 Inhibition of Na-K ATPase and K^+-stimulated 4-nitrophenylphosphatase activities by 5,5' dithiobis (2-nitrobenzoic acid) (abbreviated: Nbs_2) is carried out in a medium containing: 100 µg protein^{-1}.ml^{-1}, 2 mM CDTA, 25 mM imidazole-HCl (pH 7.5) and 5,5' dithiobis (2-nitrobenzoic acid) at the stated concentrations. After 60 min incubation at 37°C the reaction mixture is diluted 30-fold, and aliquots are assayed for Na-K ATPase and K-NPPase phosphatase activities as described in sections 2.3 (method I) and 2.7.

6.3.2 Effects on partial reactions of the Na-K ATPase system

Treatment with 5,5' dithiobis (2-nitrobenzoic acid) also inhibits the partial reactions of the Na-K ATPase system. The Na^+-stimulated ATPase activity, which is apparent at micromolar ATP concentrations (Neufeld and Levy, 1969; Post et al., 1972), is inhibited to the same extent as the overall activity (69 ± 6.7 vs. 69 ± 4.6% inhibition, 3 experiments). The same is the case for the phosphorylation by ATP in the presence of Na^+ and Mg^{2+} ions (fig. 6.6).

The K-NPPase activity is also inhibited after treatment of the enzyme with 5,5' dithiobis (2-nitrobenzoic acid), but to a lesser extent than the overall activity. This is true at various concentrations of the reagent (see fig. 6.3), as well as at different pH values of the preincubation

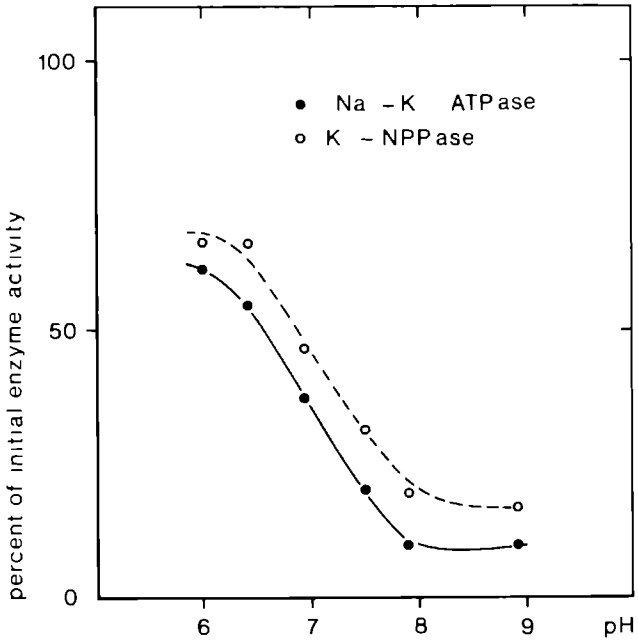


Fig. 6.4 Effect of pH on inhibition by 5,5' dithiobis (2-nitrobenzoic acid). The preincubation medium contains: 100 µg protein.ml^{-1}, 60 µM 5,5' dithiobis (2-nitrobenzoic acid), 2 mM CDTA and 50 mM Tris-maleate buffer at the indicated pH values. After 60 min reaction at 37°C, the inhibition is stopped by 30-fold dilution in 25 mM imidazole-HCl (pH 7.5). Aliquots are assayed for Na-K ATPase and K-NPPase activities after preincubation with 5,5' dithiobis (2-nitrobenzoic acid) as described in sections 2.3 (method I) and 2.7. Corrections are made for the spontaneous inactivation of the enzyme at each pH value.

medium (see fig. 6.4). Addition of ATP to the preincubation medium antagonizes the inhibitory effect of 5,5' dithiobis (2-nitrobenzoic acid) on the K-NPPase activity, primarily under non-phosphorylating conditions but also under phosphorylating conditions (Table 6.1). NPP partially prevents the inhibition under phosphorylating conditions i.e. in the presence of Mg^{2+} with or without K^{+} ions, as well as under non-phosphorylating conditions (Table 6.1).

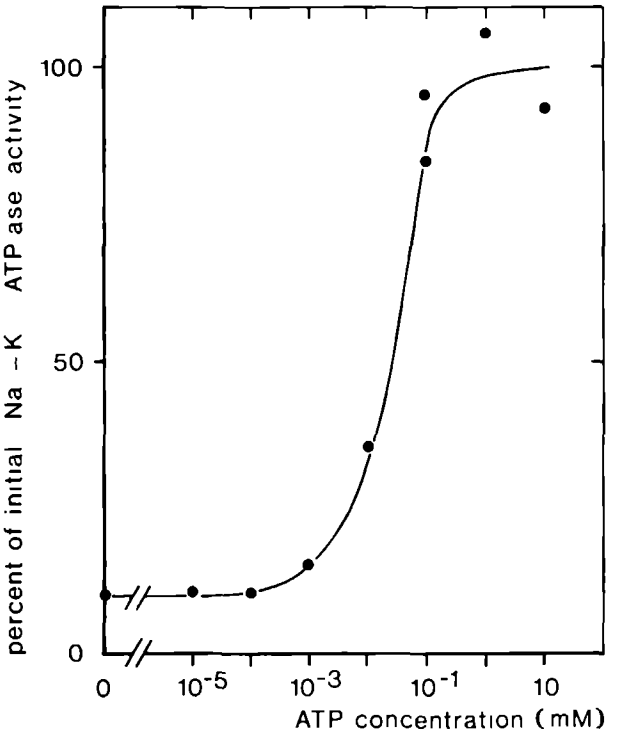


Fig. 6.5 Effect of ATP on inhibition of Na-K ATPase activity by 5,5' dithiobis (2-nitrobenzoic acid). The reaction with 5,5' dithiobis (2-nitrobenzoic acid) is carried out for 60 min at 37°C in a medium containing: 100 µg protein.ml^{-1}, 240 µM 5,5'dithiobis (2-nitrobenzoic acid), 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA and ATP (Tris salt) in the indicated concentrations. The reaction mixture is diluted 100-fold, and aliquots are assayed for Na-K ATPase activity as described in section 2.3 (method I).

6.3.3 Effects of Na^+, K^+ and Mg^{2+} ions on inhibition by 5,5' dithiobis (2-nitrobenzoic acid)

Addition of certain cations to the 5,5' dithiobis (2-nitrobenzoic acid) reaction mixture may influence the extent of Na-K ATPase inhibition. These effects are shown in fig. 6.7 a and b. Increasing the ionic strength through addition of choline chloride results in augmentation of the inhibition. The effects of addition of NaCl do not differ significantly from the ionic strength effects on the inhibition. Addition of KCl results in a small but significant reduction of the inhibition at 1 mM KCl, whereas

Table 6.1

EFFECT OF VARIOUS LIGANDS ON INHIBITION BY 5,5' DITHIOBIS (2-NITROBENZOIC ACID)

Ligands added during reaction	Residual activity	
	K-NPPase	Na-K ATPase
	%	%
Control (no 5,5' dithiobis (2-nitrobenzoic acid))	=100	=100
4 mM CDTA	12 ± 1.5	1.2 ± 1.2
10 mM ATP + 4 mM CDTA	98 ± 10	75 ± 5
10 mM ATP + 100 mM NaCl + 5 mM $MgCl_2$	64 ± 8	8 ± 2.4
10 mM NPP + 4 mM CDTA	23 ± 1	4.9 ± 0.2
10 mM NPP + 5 mM $MgCl_2$	26 ± 5	2.8 ± 0.1
10 mM NPP + 5 mM $MgCl_2$ + 10 mM KCl	36 ± 9	4.0 ± 0.6

Treatment with 5,5' dithiobis (2-nitrobenzoic acid) is performed in a medium containing. 240 µM 5,5' dithiobis (2-nitrobenzoic acid) (except for the control), 25 mM imidazole-HCl (pH 7 5), 100 µg protein.ml^{-1} and various ligands, as indicated in the table, for 60 min at 37°C. After reaction with 5,5' dithiobis (2-nitrobenzoic acid) the ligands are removed by gel filtration on a Sephadex G25 column, and aliquots are assayed for K-NPPase and Na-K ATPase activities as described in sections 2.3 (method I) and 2.7. Results from two experiments, carried out in duplicate, are presented as means with standard errors.

at higher KCl concentrations there is an increase in inhibition. This increase is significantly higher than that caused by increased ionic strength. Addition of Mg^{2+} ions leads to an increased inhibition, which is significantly higher than that due to ionic strength effect.

The effects of addition of certain combinations of cations to the 5,5' dithiobis (2-nitrobenzoic acid) reaction mixture on the inhibition have also been studied. Addition of 10 mM $MgCl_2$ with either Na^+ or K^+ ions antagonizes the increase in inhibition due to the presence of 10 mM $MgCl_2$ alone (fig. 6.8a vs. fig. 6.7b). The effect of addition of K^+ ions reaches a maximal extent at 5 mM, while in the case of Na^+ ions there is a gradually decreasing inhibition. Addition of Mg^{2+} ions in varying concentration in the presence of a fixed 100 mM NaCl concentration results in an increased inhibition (fig. 6.8b). However, in this case the Mg^{2+} concentration required for a half-maximal effect is higher than that in

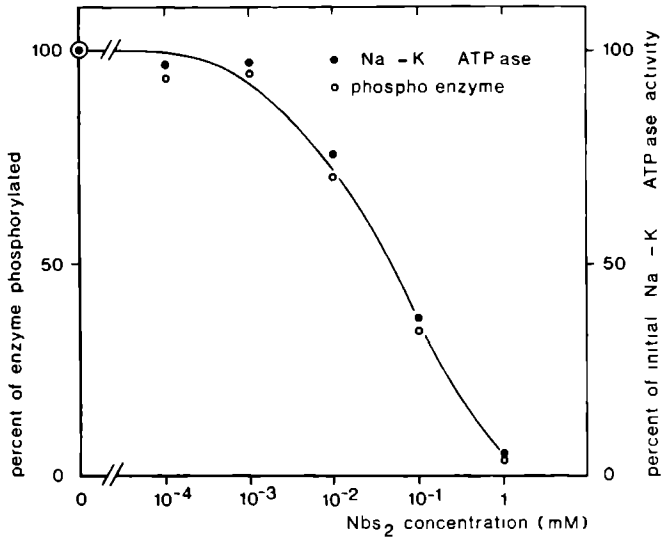


Fig. 6.6 Inhibition of phosphorylation and Na-K ATPase activity by 5,5' dithiobis (2-nitrobenzoic acid). Preincubation with 5,5' dithiobis (2-nitrobenzoic acid) (abbreviated Nbs_2) is carried out for 60 min at 37°C in a medium containing: 200 µg protein.ml^{-1}, 2 mM CDTA, 25 mM imidazole-HCl (pH 7.5), and 5,5' dithiobis (2-nitrobenzoic acid) at the indicated concentrations. The preincubation is ended by 4-fold dilution in the appropriate phosphorylation buffer, and phosphorylation (o) and Na-K ATPase activity (•) are assayed as described in sections 2.3 (method I) and 2.5.

the case of addition of Mg^{2+} ions alone (cf. fig. 6.9b with fig. 6.8b).

6.3.4 Effects of ADP and inorganic phosphate

Addition of ATP under non-phosphorylation conditions will induce the E_1 conformation of the enzyme (see fig. 1.1). The same is true for ADP, which does not phosphorylate the enzyme but binds to it with the same affinity as ATP (Nørby and Jensen, 1971). When ADP is added to the 5,5' dithiobis (2-nitrobenzoic acid) reaction mixture at a concentration that will saturate the high affinity binding site (10 µM), little or no effect of addition of NaCl on the 5,5' dithiobis (2-nitrobenzoic acid) inhibition rate is observed (fig. 6.9).

Addition of P_i at various concentrations, combined with 10 mM $MgCl_2$, does affect the inhibition. There is an increasingly diminishing inhibition

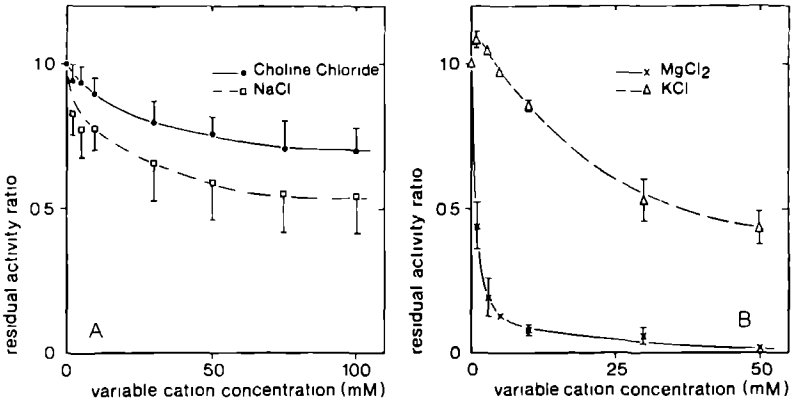


Fig. 6.7 *Effects of various cations on inhibition of* Na-K ATPase *activity* by 5,5' dithiobis (2-nitrobenzoic acid). The reaction mixture for treatment with 5,5' dithiobis (2-nitrobenzoic acid) contains: 20 µM 5,5' dithiobis (2-nitrobenzoic acid), 2 mM CDTA (omitted on the addition of Mg^{2+} ions), 25 mM imidazole-HCl (pH 7.5), 100 µg protein.ml^{-1} and the chloride of the cation at the stated concentrations. After incubation (at 37°C) during 60 min the mixture is diluted 30-fold, and is then assayed for Na-K ATPase activity as described in section 2.3 (method I). The ratio of the residual activities is plotted. At the ratio 1.0 the residual activity is 65%.
A: effects of choline chloride (—•—) and NaCl (-·-□-·-).
B: effects of KCl (---Δ---) and $MgCl_2$ (—x—).

in the presence of P_i concentrations above 0.1 mM (fig. 6.10).

6.4 Discussion

Na-K ATPase, isolated from rabbit kidney outer medulla, can be inactivated by treatment with the sulfhydryl reagents 5,5' dithiobis (2-nitrobenzoic acid) and N-ethylmaleimide. In both cases the inhibition reaction obeys second order kinetics, indicating that inhibition results from the reaction of one molecule of reagent with one vital sulfhydryl group. This does not, however, exclude the existence of more than one of these groups in the enzyme molecule.

Reaction with each of the two reagents results in parallel and equal inhibition of the Na^+-stimulated ATPase activity, the phosphorylation by ATP and the Na-K ATPase activity. Binding of ATP to the enzyme protects against inactivation by either reagent. The ATP concentration required for 50% protection against complete inhibition by 5,5' dithiobis (2-nitro-

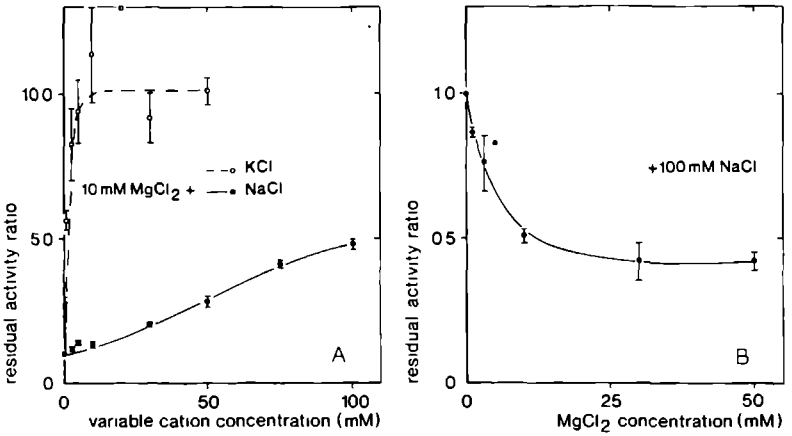


Fig. 6.8 Effects of combinations of cations on the inhibition of Na-K ATPase activity by 5,5' dithiobis (2-nitrobenzoic acid). The reaction with 5,5'dithiobis (2-nitrobenzoic acid) is performed in a medium containing 20 µM 5,5' dithiobis (2-nitrobenzoic acid), 25 mM imidazole-HCl (pH 7.5), 100 µg protein.ml^{-1} and chlorides of the cations at the stated concentrations. After 60 min incubation at 37°C the mixture is diluted 30-fold and assayed for Na-K ATPase activity as described in section 2.3 (method I). The results are plotted as the ratio of residual activities.

A. Cations present are 10 mM Mg^{2+} plus varying concentrations of Na^+ (—•—) or KCl (-·-o-·-). At the ratio 1.0 the residual activity is 5%.

B. Cations present are 100 mM Na^+ plus varying concentrations of Mg^{2+}. At the ratio 1.0 the residual activity is 45%.

benzoic acid) amounts to 20 µM, in good agreement with the value of 15 µM calculated from fig. 2 in Patzelt-Wenczler et al. (1975). The dissociation constant for ATP binding to the site, which it protects against reaction with 5,5' dithiobis (2-nitrobenzoic acid) is 5 µM, calculated according to the method of Carter et al. (1968) from the data in fig. 6.6. The corresponding dissociation constant for ATP for protection against N-ethylmaleimide inhibition amounts to 20 µM, when calculated from the data in fig. 5.3. These values approach the K_{diss} values of ca 0.2 µM for the binding of ATP (Nørby and Jensen, 1971; Hegyvary and Post, 1971), suggesting that ATP protects in both cases through binding to the high affinity site of the enzyme. This may occur either by shielding a sulfhydryl group in or near the ATP binding center or else by inducing a conformational change of the enzyme leading to the shielding of a vital sulfhydryl group elsewhere.

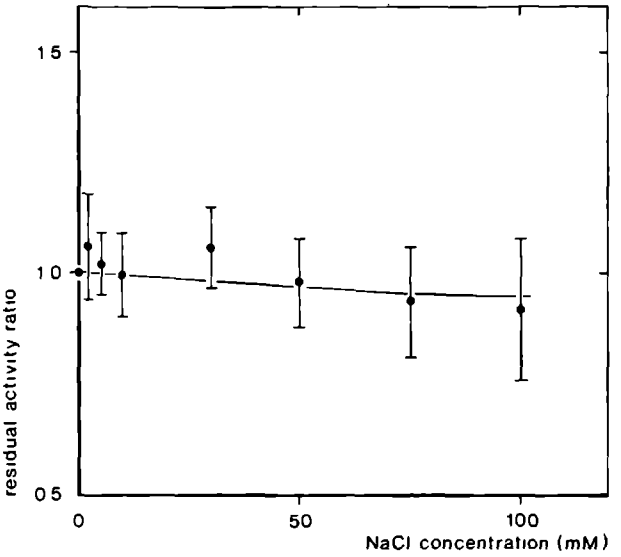


Fig 6.9 Effects of ADP plus NaCl on the inhibition of Na-K ATPase activity by 5,5' dithiobis (2-nitrobenzoic acid) The reaction mixture for treatment with 5,5' dithiobis (2-nitrobenzoic acid) contains 20 µM 5,5' dithiobis (2-nitrobenzoic acid), 2 mM CDTA, 10 µM ADP, 25 mM imidazole-HCl (pH 7.5), 100 µg protein.ml^{-1} and NaCl at the stated concentrations. After reaction for 60 min at 37°C the mixture is diluted 30-fold, and assayed for Na-K ATPase activity as described in section 2.3 (method I). The results are plotted as the residual activity ratio. At the ratio 1.0 residual activity is 80%.

There is some evidence favouring the former alternative, viz. the localization of a vital sulfhydryl group in the active center. First, there is qualitative agreement between the effects of 5,5' dithiobis (2-nitrobenzoic acid) and of the ATP binding site-directed sulfhydryl reagent S- 2,4 dinitrophenyl 6-mercaptopurine riboside triphosphate (Patzelt-Wenczler et al., 1975) on the Na-K ATPase and K-NPPase activities. Secondly prior treatment with 5,5' dithiobis (2-nitrobenzoic acid) has an effect on the reaction rate of butanedione with an arginine residue situated in the ATP binding center (section 8.3.4).

There are also differences between the effects of 5,5' dithiobis (2-nitrobenzoic acid) and those of N-ethylmaleimide. First, after reaction with N-ethylmaleimide the K-NPPase activity is inhibited to the same extent as the Na-K ATPase activity, whereas after reaction with 5,5' dithiobis

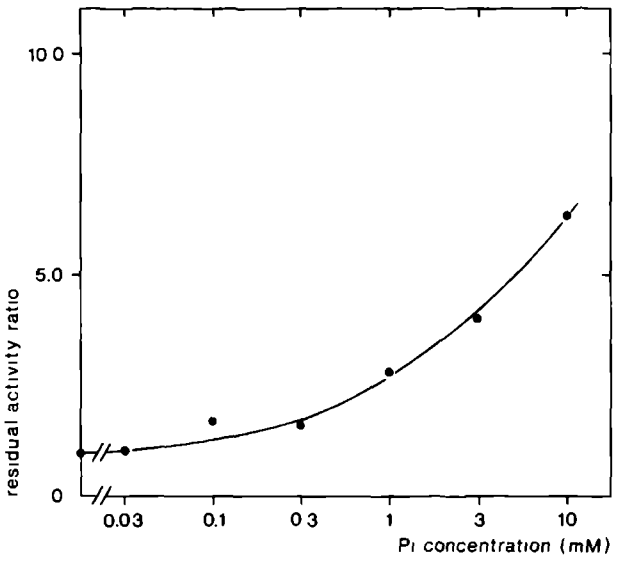


Fig. 6.10 Effects of inorganic phosphate plus Mg^{2+} on the inhibition of Na-K ATPase activity by 5,5' dithiobis (2-nitrobenzoic acid). The reaction medium for treatment with 5,5' dithiobis (2-nitrobenzoic acid) contains: 20 µM 5,5'dithiobis (2-nitrobenzoic acid), 10 mM $MgCl_2$, 25 mM imidazole-HCl (pH 7.5), 100 µg protein.ml^{-1} and P_i at the stated concentrations. After reaction during 60 min at 37°C the mixture is diluted 30-fold and assayed for Na-K ATPase activity as described in section 2.3 (method I). The results are plotted as the residual activity ratio. At the ratio 1.0 the residual activity is 4%.

(2-nitrobenzoic acid) the phosphatase activity is significantly less inhibited. Secondly, 4-nitrophenylphosphate antagonizes the inhibition of the Na-K ATPase activity by N-ethylmaleimide, but not that by 5,5' dithiobis (2-nitrobenzoic acid). Thirdly, the protecting effect of ATP on the inactivation of the phosphatase activity occurs in the case of N-ethylmaleimide under non-phosphorylating conditions only, whereas in the case of 5,5' dithiobis (2-nitrobenzoic acid) protection occurs both under phosphorylating and under non-phosphorylating conditions. Fourthly, the same is true for the antagonizing effect of 4-nitrophenylphosphate. Finally, the presence of certain cations (Na^+, K^+, Mg^{2+}) has different effects on the inhibition by the two reagents (sections 5.3.3 and 6.3.3).

In view of the biphasic effects of the cations on the inhibition by the two reagents, particularly N-ethylmaleimide, it appears that conformational changes of the enzyme are responsible for the cation effects. The

conformation of the enzyme will determine the exposure of the essential sulfhydryl group(s). From the various cation effects on the inhibition by the two sulfhydryl reagents and from other published data, it appears that at least four different enzyme conformations occur, which are determined by the presence of either CDTA, NaCl, KCl or $MgCl_2$.

The CDTA conformation may be considered as the reference conformation, which is changed to one of the others by the addition of a cation. The effects of addition of NaCl and KCl are opposed to each other. KCl decreases and NaCl enhances the inhibition by N-ethylmaleimide, KCl affects the inhibition by 5,5' dithiobis (2-nitrobenzoic acid), while NaCl has no effect. This suggests that the enzyme has two different conformations in the presence of either NaCl or KCl. Jørgensen (1975b) came to the same conclusion on the basis of his tryptic digestion experiments. Addition of $MgCl_2$ results in increased inhibition by N-ethylmaleimide as well as by 5,5' dithiobis (2-nitrobenzoic acid), effects which are completely different from those caused by NaCl or KCl. This indicates a fourth conformational state of the enzyme, which is induced by $MgCl_2$. Such a conformational change due to addition of $MgCl_2$ has also been suggested by Kuriki et al (1977) on the basis of calorimetric findings.

Combined addition of $MgCl_2$ with either NaCl or KCl shows no additive effects with either reagent. Hence, there appears to occur competition between Mg^{2+} and either Na^+ or K^+ ions for each of the ion-induced conformational states of the enzyme (for a similar competition between Mg^{2+} and Na^+ ions. see Robinson, 1970). These ion-induced conformation changes may explain the different effects of addition of either Mg^{2+}, Na^+ or K^+ ions on the dissociation rate constant for the enzyme-ouabain complex formed in the presence of $MgCl_2$ and P_i with Na-K ATPase from rabbit kidney cortex (Schuurmans Stekhoven et al., 1976b) The latter experiments also demonstrate the non-additivity of the effects of $MgCl_2$ and either NaCl or KCl on the dissociation rate constant.

Various values for the affinity of either Na^+, K^+ or Mg^{2+} ions to Na-K ATPase have been reported (see section 1.3). These affinities are rather high (K_{diss} ca. 1 mM), which suggests that binding of the cations to these sites can explain only the low concentration phase of the biphasic effects. The high concentration phase may be due to effects of the cation on binding sites with a lower affinity such as phospholipids (Kanlike et

al., 1976) or another cation binding site.

The difference between the effects of a cation on the inhibition by N-ethylmaleimide and 5,5' dithiobis (2-nitrobenzoic acid) suggests that different essential group(s) are involved in the reaction with either reagent and that these may be located in different places.

So far we have only discussed the sulfhydrylgroups, which are essential for the Na-K ATPase activity. There appears to exist an additional essential group, which is primarily involved in the K-NPPase activity. This is suggested by the fact that the K-NPPase activity is protected by ATP and NPP from inhibition by 5,5' dithiobis (2-nitrobenzoic acid) under phosphorylating as well as non-phosphorylating conditions, whereas the Na-K ATPase activity is only protected under non-phosphorylating conditions.

In conclusion, we may state that at least two (possibly three) different essential sulfhydryl groups are present in Na-K ATPase, one (possibly two) of which are involved in the inhibition of Na-K ATPase activity by 5,5' dithiobis (2-nitrobenzoic acid) while one is involved in the inhibition by N-ethylmaleimide.

CHAPTER 7

CLASSIFICATION OF SULFHYDRYL GROUPS

7.1 Introduction

In chapters 5 and 6 we have shown that Na-K ATPase is inhibited after reaction with N-ethylmaleimide as well as with 5,5' dithiobis (2-nitrobenzoic acid). Although both compounds react with sulfhydryl groups, differences in their effects on the partial reactions and in the effects of cations on their inhibitory effects are observed. This has led us to the assumption that there are at least three different essential sulfhydryl groups in the enzyme. In this chapter we shall attempt to determine the number and reactivity of the sulfhydrylgroups that are modified. The distribution of the sulfhydryl groups over the two subunits of the enzyme will also be determined.

7.2 Methods

7.2.1 Determination of sulfhydryl groups with 5,5' dithiobis (2-nitrobenzoic acid)

This is carried out by reading the 412 nm absorbance of 5-thio-2-nitrobenzoic acid, which is released during reaction of the enzyme with the parent reagent. A mixture of 1 mM 5,5' dithiobis (2-nitrobenzoic acid), 400 µg protein.ml^{-1}, 25 mM imidazole-HCl (pH 7.5), with or without 1% (w/w) sodium dodecyl sulfate, is incubated for 60 min at 37^{o}C. Thereafter, the insoluble protein is removed by centrifugation for 15 min at 20,000g, and the 412 nm absorbance of the supernatant is determined. Calibration is achieved by reading the absorbance after addition of known amounts of cysteine-HCl instead of enzyme to the reaction mixture. Corrections are made for the absorbance of imidazole and protein. The number of sulfhydryl groups determined in the presence of 1% sodium dodecyl sulfate is referred to as 'total sulfhydryl groups'. The number of sulfhydryl groups per molecule Na-K ATPase is calculated by assuming a molecular weight of

250.000 (Kepner and Macey, 1968).

7.2.2 Determination of the partition coefficients of the sulfhydryl reagents

The partition coefficient of N-ethylmaleimide is determined by mixing 1 ml of a solution of 10 mM N-ethylmaleimide in 25 mM imidazole-HCl (pH 7.5) with 10 ml water-saturated octanol-1. After separation of the two phases, the N-ethylmaleimide concentration is determined in aliquots of both phases. The aliquot of the aqueous layer is diluted 100-fold and mixed with 0.1 vol. 100 µM cysteine-HCl, 25 mM imidazole-HCl (pH 7.5). The aliquot of the octanol layer is mixed with 2 vols of this cysteine solution. After incubation for 60 min at 37°C, remaining cysteine is determined by addition of an equal volume of 5 mM 5,5' dithiobis (2-nitrobenzoic acid) and further incubation for 60 min at 37°C. The 412 nm absorbance is then determined in the water layer. The concentration of N-ethylmaleimide can be calculated from the decrease in the amount of cysteine.

The partition coefficient for 5,5' dithiobis (2-nitrobenzoic acid) is determined in a similar way, except that 5 vols. octanol layer are mixed with 1 vol. of the cysteine solution. Calibration curves are obtained by reading the 412 nm absorbance of solutions of known concentrations of cysteine and excess 5,5' dithiobis (2-nitrobenzoic acid) in 25 mM imidazole (pH 7.5) buffer.

7.2.3 Determination of tritiated protein

The method described by Albanese and Goodman (1977) is used. Na-K ATPase preparations are treated with (^{3}H) N-ethylmaleimide (tritiated in the ethyl group, 150-200 Ci.mmol^{-1}, NEN Chemicals, Frankfurt a.M., W-Germany) under native conditions as well as after solubilization in SDS. The treated preparations are subjected to SDS polyacrylamide gel electrophoresis as described in section 2.9.2 (Laemmli system). After gel electrophoresis, the gel is cut in 1.5 mm slices. The slices are weighed in counting vials and dissolved in a 10 : 1 (v/v) mixture of NH_4OH (conc.) and H_2O_2 (conc.). After several days at room temperature, when the slices are dissolved, 10 ml scintillation fluid (Aquasol) is added and the mixture is counted for ^{3}H in a liquid scintillation analyzer. The weight of the gel

slices is used for calculation of the relative mobility of the radioactivity. A parallel gel is stained with Coomassie blue, in order to determine the position of the protein bands.

7.2.4 Determination of number of alkylated groups

The number of groups alkylated by (^{3}H) N-ethylmaleimide is determined after incubation of Na-K ATPase with (^{3}H) N-ethylmaleimide at 37°C in a medium containing 25 mM imidazole-HCl (pH 7.5), 1 mg protein.ml^{-1}, varying concentrations of N-ethylmaleimide and other additives as stated. The varying N-ethylmaleimide concentrations are prepared by mixing (^{3}H) N-ethylmaleimide with various amounts of N-ethylmaleimide so that the sum of their concentrations,determining the final total N-ethylmaleimide concentration, has the desired value. Over a period of 30 minutes, 30-µl samples are taken at 5-min intervals and diluted in 1 ml 25 mM imidazole-HCl (pH 7.5) and 2 mM DTE, at 0°C. After stopping the reaction of N-ethylmaleimide, the residual Na-K ATPase activity is determined in an aliquot of the stopped reaction mixture; the remainder is denatured by addition of 1 ml 10% TCA, 1 mM N-ethylmaleimide. The denatured protein is removed by filtration over a Selectron AE95 filter (1.2 µM pore size) and washed 5 times with 5 ml cold 5% TCA (w/v). Filters are dissolved in 10 ml Aquasol, and analyzed for ^{3}H content in a liquid scintillation analyzer. Blanks are prepared by denaturing the protein before addition of (^{3}H) N-ethylmaleimide, followed by filtration and washing as described above. The number of molecules N-ethylmaleimide bound per molecule Na-K ATPase is calculated by assuming a molecular weight of 250,000 for the Na-K ATPase complex (Kepner and Macey, 1968).

7.2.5 Various methods

Purified Na-K ATPase. The purified enzyme preparation is obtained from rabbit kidney outer medulla microsomes as described in section 3.4. The purified preparation is washed as described in section 5.2 before modification.

Enzyme activities. Na-K ATPase activity is determined as described in section 2.3 (method I).

Treatment with sulfhydryl reagents. This is performed with washed purified Na-K ATPase as described in section 5.2 for N-ethylmaleimide and

in section 6.2 for 5,5' dithiobis (2-nitrobenzoic acid).

Protein determination. Protein is determined as described in section 2.9.1.

7.3 Results

7.3.1 Number of sulfhydryl groups reacting with the two reagents

We have determined the number of sulfhydryl groups able to react with 5,5' dithiobis (2-nitrobenzoic acid) in completely inactivating concentration (1 mM) after preincubation with various concentrations of N-ethylmaleimide. This titration is carried out in the presence or absence of 1% (w/w) sodium dodecyl sulfate. The total number of sulfhydryl groups, that can be titrated per molecule of Na-K ATPase (MW 250,000) in the presence of sodium dodecyl sulfate is 36 ± 2.0. In the absence of sodium dodecyl sulfate, 5,5' dithiobis (2-nitrobenzoic acid) reacts with 12 ± 1.4 sulfhydryl groups.

When the enzyme has previously been treated with 10 mM N-ethylmaleimide, which is a nearly completely inhibitory concentration, 5,5' dithiobis (2-nitrobenzoic acid) does not react with the enzyme in the absence of sodium dodecyl sulfate. In the presence of the latter substance, 5,5' dithiobis (2-nitrobenzoic acid) reacts with 10 ± 1.7 of the 36 sulfhydryl groups, suggesting that N-ethylmaleimide has reacted with 36 - 10 = 26 sulfhydryl groups. Intermediate results are obtained when the enzyme is preincubated with 1 mM instead of 10 mM N-ethylmaleimide (Table 7.1).

7.3.2 Effect of consecutive treatments with both sulfhydryl reagents

To investigate whether 5,5' dithiobis (2-nitrobenzoic acid) reacts with the same functional groups as N-ethylmaleimide does, we have applied these reagents consecutively. Subsequently we have used 1,4 dithioerythritol to reverse the reaction with 5,5' dithiobis (2-nitrobenzoic acid). The reaction of N-ethylmaleimide is not reversed by the latter treatment (see chapter 5).

Incubation with 1,4 dithioerythritol after preincubation with 5,5' dithiobis (2-nitrobenzoic acid) leads to considerable reversal of the inhibition caused by 5,5' dithiobis (2-nitrobenzoic acid) (Table 7.2). When after preincubation with 5,5' ditiobis (2-nitrobenzoic acid) the

Table 7.1

SULFHYDRYL GROUPS AFTER REACTION WITH N-ETHYLMALEIMIDE

Concentration N-ethylmaleimide during preincubation	Residual Na-K ATPase activity	Free sulfhydryl groups per mol enzyme	
mM	%	no SDS	in 1% SDS
0	- 100	12 ± 1.4	36 ± 2.0
1	55 ± 5	6 ± 0.7	25 ± 3.5
10	3.3 + 0.4	0	10 ± 1.7

Treatment with N-ethylmaleimide is carried out in a medium containing: N-ethylmaleimide (0, 1 or 10 mM), 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA and 4 mg protein.ml^{-1}, for 30 min at 37°C. Excess N-ethylmaleimide is removed by gel filtration on a Sephadex G25 column. Aliquots of the eluate are assayed for sulfhydryl group content and Na-K ATPase activity as described in sections 2.3 (method I) and 7.2.1. Results are the means with standard errors of three experiments, except for the value of 36 + 2.0 sulfhydryl groups, which represents eight determinations. Mean specific activity of the preparations used in 1336 (S.E. 140) µmol ATP split.mg protein^{-1}.hr^{-1}.

Table 7.2

EFFECT OF 5,5' DITHIOBIS (2-NITROBENZOIC ACID) ON INHIBITION BY N-ETHYLMALEIMIDE

Additives during preincubation			Residual activity Na-K ATPase	(n)
I	II	III	%	
-	-	DTE	100	5
5,5' dithio-bis (2-nitro-benzoic acid)	-	-	30.6 ± 3	4
ibid.	-	DTE	75.0 ± 7.2	5
ibid.	N-ethyl-maleimide	DTL	22.2 ± 4	5
ibid.	N-ethyl-maleimide + ATP	DTE	51.5 ± 6.3	2

Preincubation I is carried out in a medium containing: 0 or 240 µM 5,5' dithiobis (2-nitrobenzoic acid), 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA, 100 µg protein.ml^{-1} at 37°C for 60 min. The mixture is then applied to a Sephadex G25 column, to remove excess 5,5' dithiobis (2-nitrobenzoic acid). Preincubation II is performed in a medium containing: 0 or 10 mM N-ethylmaleimide, 0 or 10 mM ATP, 25 mM imidazole-HCl (pH 7.5), 4 mM CDTA, 10 µg protein.ml^{-1} for 30 min at 37°C. Preincubation III is performed with 10 mM dithioerythritol for 30 min at 37°C. Aliquots are assayed for Na-K ATPase activity. Results are presented as means with standard errors, n is the number of experiments.

enzyme is treated with N-ethylmaleimide, incubation with 1.4 dithioerythritol does not restore the original activity. This suggests that N-ethylmaleimide can react with additional vital groups in the enzyme complex after certain essential sulfhydryl groups have previously been blocked with 5,5' dithiobis (2-nitrobenzoic acid). Table 7.2 also shows that in this case the effect of N-ethylmaleimide can be antagonized at least in part, by addition of 10 mM ATP.

Taken together with the findings in the previous section, this means that at least 26 of the 36 sulfhydryl groups per enzyme molecule react with N-ethylmaleimide, whereas max. 10 do not react. From these 26 residues at least 12 can also be modified by 5,5' dithiobis (2-nitrobenzoic acid). At least one residue of the latter 12 and at least one of the 14 other residues is vital for the activity of the enzyme. The reaction between each of these residues and the respective sulfhydryl reagents can be prevented by ATP.

7.3.3 Reaction of N-ethylmaleimide with the subunits

The distribution of groups reacting with N-ethylmaleimide over the two subunits of Na-K ATPase depends on the reaction conditions. Under native conditions (^{3}H) N-ethylmaleimide reacts only with groups situated on the catalytic subunit (fig. 7.1). When the enzyme is dissolved in 10% SDS (w/v) prior to reaction with (^{3}H) N-ethylmaleimide, reactive groups are also demonstrated in the glycoprotein subunit. From the partition of tritium over both subunits after reaction with dissolved enzyme, we calculate that 34 out of the total 36 sulfhydryl groups are localized on the catalytic subunit and only 2 on the glycoprotein subunit.

7.3.4 Number of groups modified by (^{3}H) N-ethylmaleimide and residual Na-K ATPase activity

The number of groups modified by (^{3}H) N-ethylmaleimide and the residual Na-K ATPase activity are determined after modification in a medium containing 25 mM imidazole-HCl (pH 7.5), 1 mg.ml^{-1} protein, 0-5 mM (^{3}H) N-ethylmaleimide and various additions. Fig. 7.2 refers to an addition of 2 mM CDTA, fig. 7.3 to addition of 10 mM $MgCl_2$ or 100 mM NaCl, fig. 7.4A to addition of 5 mM $MgCl_2$ + 100 mM NaCl and fig. 7.4B to addition of 5 mM $MgCl_2$ + 100 mM NaCl + 6 mM AMPPNP. For better comparison the number of groups modified at a certain percentage inhibition are listed in table 7.3.

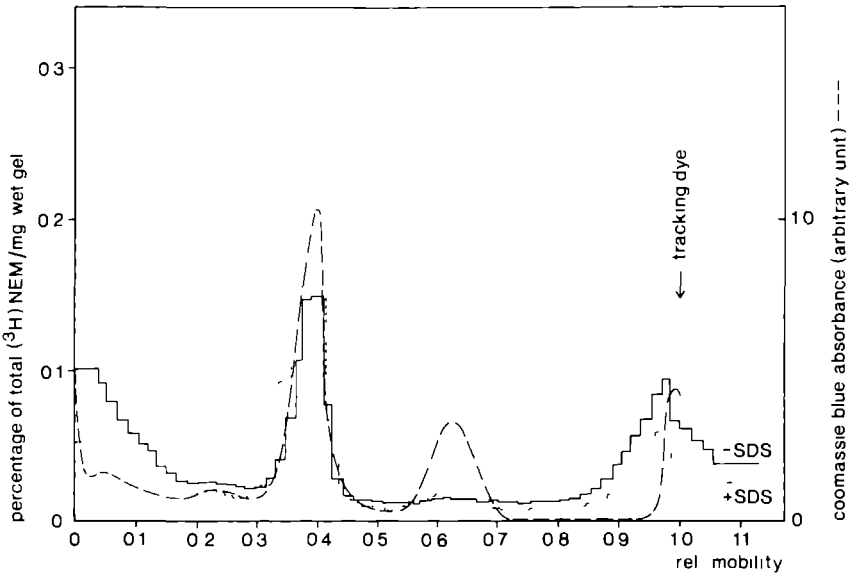


Fig. 7.1 Gel electrophoretic pattern of (^{3}H) N-ethylmaleimide labeled Na-K ATPase. Reaction of Na-K ATPase with (^{3}H) N-ethylmaleimide during 30 min at 37°C is carried out in a medium containing: 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA, 10 mM (^{3}H) N-ethylmaleimide (specific radioactivity 50 Ci.mmol^{-1}), 1 mg protein.ml^{-1} with (----) or without (——) 1% SDS (w/v). After reaction excess (^{3}H) N-ethylmaleimide is removed by gel filtration over a Sephadex G25 coarse column (100 x 5 mm). When necessary, the protein is then dissolved in 1% SDS (w/v). SDS polyacrylamide gel electrophoresis is performed according to Laemmli (1969). After electrophoresis the gels are sliced and the amount of ^{3}H is determined. A control gel is stained with Coomassie blue (— — —).

The number of modified groups gradually increases with the inhibition of the enzyme, after a certain lag phase. The maximal number of 26 groups modified by N-ethylmaleimide is never reached in these experiments. This is probably entirely due to the fact that at a high N-ethylmaleimide concentration the specific radioactivity of (^{3}H) N-ethylmaleimide is too low to allow reliable determinations.

The number of groups modified at the same residual Na-K ATPase activity increases in the following order of the additions to the modification medium: $MgCl_2$ < NaCl ≃ CDTA < NaCl + $MgCl_2$ ≃ NaCl + $MgCl_2$ + AMPPNP.

Table 7.3

NUMBER OF GROUPS MODIFIED BY (^{3}H) N-ETHYLMALEIMIDE IN THE PRESENCE OF VARIOUS ADDITIONS

percent residual Na-K ATPase activity	Number of modified groups per mol Na-K ATPase in the presence of:				
	$MgCl_2$	NaCl	CDTA	NaCl + $MgCl_2$	NaCl + $MgCl_2$ + AMPPNP
90	3.6	3.6	3.5	6.2	6.8
80	4.6	4.8	4.5	7.0	6.9
70	5.6	6.1	6.0	7.8	7.3
60	6.5	7.3	7.5	8.2	8.0
50	7.5	8.5	9.5	8.8	8.6
40	8.5	9.5	-	9.3	9.8
30	9.6	11.5	-	10.0	11.0
20	11.0	13.5	-	11.8	13.0
10	13.0	16.8	-	16.0	15.3

The number of modified groups is calculated from the curves in figs. 7.2, 7.3 and 7.4.

7.3.5 Partition coefficients of the sulfhydryl reagents

The partition coefficients of N-ethylmaleimide and 5,5' dithiobis (2-nitrobenzoic acid) between 25 mM imidazole (pH 7.5) and octanol-1 have been determined. The observed values are: 15 for N-ethylmaleimide (buffer/octanol) and 5 x 10^3 for 5,5' dithiobis (2-nitrobenzoic acid) (buffer/octanol). The latter value is in good agreement with the value of 4 x 10^3 reported by Murphy (1976a). These values indicate that N-ethylmaleimide is much more lipophilic than 5,5' dithiobis (2-nitrobenzoic acid).

7.4 Discussion

As already shown in chapters 5 and 6, Na-K ATPase, isolated from rabbit kidney outer medulla, can be inactivated by treatment with the sulfhydryl reagents 5,5' dithiobis (2-nitrobenzoic acid) and N-ethylmaleimide. There are both similarities and differences between the effects of the two reagents (section 6.4). In this chapter two further differences are added to the list of differences.

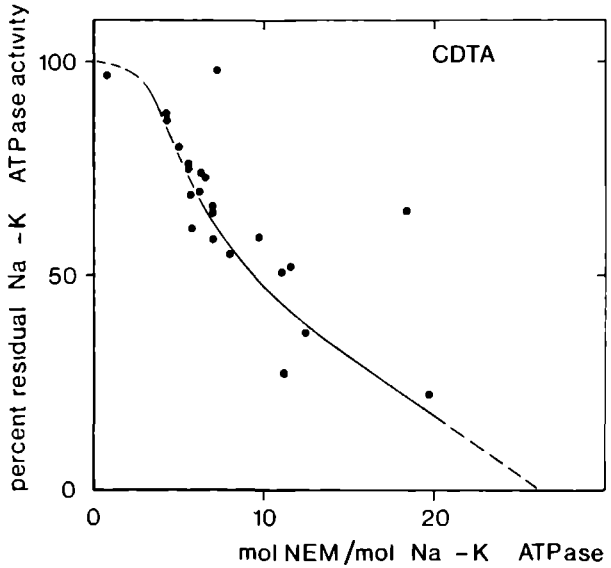


Fig. 7.2 Residual Na-K ATPase activity vs. number of N-ethylmaleimide-modified groups in the presence of 2 mM CDTA. The N-ethylmaleimide reaction mixture consists of. 25 mM imidazole-HCl (pH 7.5), 2 mM CDTA, 1 mg.ml^{-1} protein and various concentrations of (^{3}H) N-ethylmaleimide. After pre-incubation at 37°C up to 30 min, the reaction is ended by 33.3-fold dilution of 30 µl preincubation mixture with 25 mM imidazole-HCl (pH 7.5), 2 mM DTE. The residual Na-K ATPase activity and the amount of N-ethylmaleimide bound per mol Na-K ATPase are determined in aliquots of this mixture.

First, there is a difference in the number of sulfhydryl groups that can be modified by each reagent in the absence of detergent. 5,5' Dithiobis (2-nitrobenzoic acid) reacts with only 12 sulfhydryl groups, whereas N-ethylmaleimide can modify 26 out of 36 sulfhydryl groups. The total number of sulfhydryl groups of the enzyme, determined with 5,5' dithiobis (2-nitrobenzoic acid) in the presence of SDS (36 + 2) falls within the range of the numbers (51, 28 and 24) calculated from the data reported by Kyte (1972a), Hopkins et al. (1976) and Perrone et al. (1975), and agrees within the experimental error with the number (39) reported by Esmann (1977).

Secondly, a vital group for Na-K ATPase activity exists that can be modified by N-ethylmaleimide, but is not accessible to 5,5'dithiobis (2-nitrobenzoic acid). This is shown in the experiment in which the enzyme is made to react with N-ethylmaleimide after treatment with 5,5' dithiobis (2-nitrobenzoic acid). Only the inhibition by 5,5' dithiobis (2-nitrobenzoic

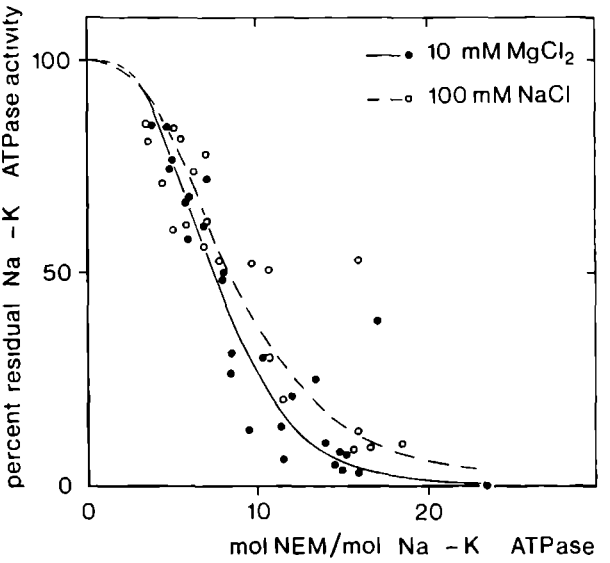


Fig. 7.3 Residual Na-K ATPase activity vs. number of N-ethylmaleimide-modified groups in the presence of either 10 mM $MgCl_2$ or 100 mM NaCl. The experiment is performed as described in the legend of fig. 7.2, exc. that 100 mM NaCl, or instead of 2 mM CDTA 10 mM $MgCl_2$ is added to the N-ethylmaleimide reaction mixture.

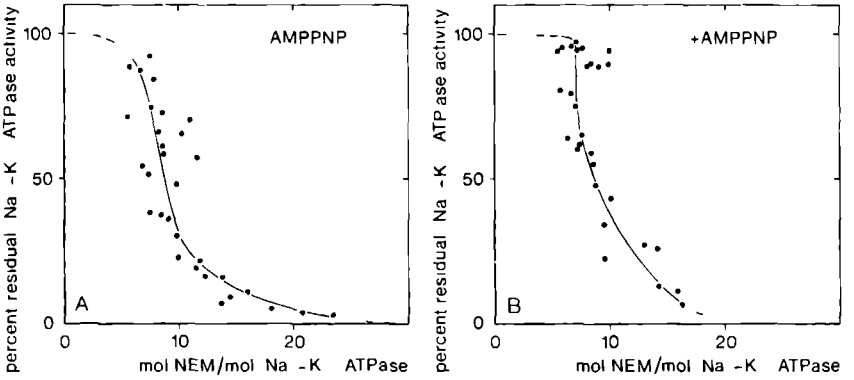


Fig. 7.4 Residual Na-K ATP activity vs. number of N-ethylmaleimide-modified groups in the presence of 5 mM $MgCl_2$ + 100 mM NaCl with or without 6 mM adenylyl imidodiphosphate (AMPPNP). The experiment is performed as described in the legend of fig. 7.2, exc. that instead of 2 mM CDTA 5 mM $MgCl_2$ + 100 mM NaCl (A) or 5 mM $MgCl_2$ + 100 mM NaCl + 6 mM AMPPNP (B) is added to the N-ethylmaleimide reaction mixture.

acid) can be reversed by subsequent treatment with dithioerythritol. However, after treatment of the 5,5' dithiobis (2-nitrobenzoic acid)-inhibited enzyme with N-ethylmaleimide, no restoration of the ATPase activity is observed anymore upon incubation with dithioerythritol. This indicates that N-ethylmaleimide has in this case reacted with a vital group, which has not been blocked by reaction with 5,5'dithiobis (2-nitrobenzoic acid).

The reaction of N-ethylmaleimide with the enzyme after prior treatment with 5,5' dithiobis (2-nitrobenzoic acid) can be partially prevented by ATP. From the degree of protection obtained by 10 mM ATP it appears that in this case a low affinity ATP binding site is involved. This may be due either to a change in affinity of the former high affinity site by prior reaction with 5,5' dithiobis (2-nitrobenzoic acid) or else to the presence of a low affinity ATP binding site, which is not modified by reaction with 5,5' dithiobis (2-nitrobenzoic acid).

It is shown by gel electrophoresis after labeling the native enzyme with (^{3}H) N-ethylmaleimide that the modified groups are all situated on the catalytic subunit. Since previous reaction between native enzyme and N-ethylmaleimide prevents reaction with 5,5' dithiobis (2-nitrobenzoic acid) (Table 7.1), it appears that N-ethylmaleimide reacts with all sulfhydryl groups on the native enzyme that are accessible for 5,5' dithiobis (2-nitrobenzoic acid). This means that all groups of the native enzyme reacting with 5,5' dithiobis (2-nitrobenzoic acid) must be located on the catalytic subunit. When the enzyme is dissolved in SDS prior to treatment with N-ethylmaleimide, alkylation of the glycoprotein subunit can also be demonstrated. From the total of 36 sulfhydryl groups present in Na-K ATPase, 34 are situated on the catalytic subunit and 2 on the glycoprotein subunit.

These results enable us to classify all sulfhydryl groups on Na-K ATPase in three different classes:

- Class A, comprising 12 sulfhydryl groups reacting with both N-ethylmaleimide and 5,5' dithiobis (2-nitrobenzoic acid), including at least one that is vital for enzyme activity. These groups are all situated on the catalytic subunit.
- Class B, comprising (at least) 14 sulfhydryl groups reacting only with N-ethylmaleimide. The groups in this class are also located on the catalytic subunit, and they include an essential group.

-Class C, comprising (at most) 10 sulfhydryl groups reacting neither with 5,5' dithiobis (2-nitrobenzoic acid) nor with N-ethylmaleimide in the native state. Nothing can be said about the presence of a vital group in this class. The sulfhydryl groups located on the glycoprotein subunit belong to this class.

From the octanol/water partition coefficients of N-ethylmaleimide and 5.5' dithiobis (2-nitrobenzoic acid) it appears that the class A sulfhydryl groups must be located on the hydrophilic surface of the enzyme, whereas the class B sulfhydryl groups seem to be present in a more hydrophobic environment. The class C sulfhydryl groups must be located in very inaccessible parts of the molecule, which are only exposed upon denaturation by sodium dodecyl sulfate.

Thus two different types of vital sulfhydryl groups are present in Na-K ATPase, a hydrophilic type reacting with both reagents and probably located in the ATP binding site (class A), and a more hydrophobic type reacting with N-ethylmaleimide only (class B) and probably not located in an active center (see section 5.4). There appear to be two different class A vital groups, one located in the high affinity ATP binding site, the other involved in the phosphatase activity (section 6.4).

The N-ethylmaleimide-sensitive essential group for the Na-K ATPase activity is not the most reactive one. It is clear that at least the equivalent of 3 groups are alkylated rapidly without loss of enzyme activity. From the observations that inhibition by N-ethylmaleimide is increased in the presence of 10 mM $MgCl_2$, relative to the inhibition in the presence of either 2 mM CDTA or 100 mM NaCl (fig. 5.9 a and b) and that at a certain degree of inhibition fewer groups are modified in the presence of $MgCl_2$ than in the presence of either 2 mM CDTA or 100 M NaCl, we may conclude that the essential sulfhydryl group at least has an increased reactivity in the presence of 10 mM $MgCl_2$. Addition of $MgCl_2$ (5 mM) to the enzyme in the presence of 100 mM NaCl, results in an increased inhibition (fig. 5.10b), but also in an increased number of modified groups. This means that the reactivity of some non-essential groups is increased, in addition to the increase in reactivity of the essential group. The increase in reactivity of the essential group accounts for the observed increase in inhibition, while the increase in reactivity of some non-essential groups explains the observation that at a certain degree of inhibition more groups are modified in the presence of 5 mM $MgCl_2$ than in the absence (both in the presence of 100 mM NaCl).

From the protecting effect of AMPPNP on the inhibition one would expect that for a certain degree of inhibition the number of modified groups will be larger in the presence of AMPPNP than in its absence. In both cases the same relation between number of modified groups and residual activity exists (fig. 7.5 a and b). This must be due to the fact that either the number of groups protected by AMPPNP is so small that it is within the experimental error, or that there is an overall decrease in the reactivity of the sulfhydryl groups by binding of AMPPNP.

Finally, we wish to point out that all changes in reactivity of sulfhydryl groups, whether caused by addition of cations or by addition of AMPPNP, are due to changes in exposure of these groups. In the native enzyme the reactive sulfhydryl groups are located on the catalytic subunit. This means that this subunit undergoes conformational changes, suggesting that it is rather flexible.

EFFECTS OF 2,3 BUTANEDIONE ON OVERALL REACTION AND PARTIAL REACTIONS

8.1 Introduction

In recent years reagents such as butanedione and phenylgyoxal, which react specifically with arginine residues, have frequently been applied in the study of enzymes. Arginine residues are often involved in the binding of negatively charged substrates or cofactors to enzyme molecules (Riordan et al., 1977).

In this chapter we report a study of the effect of the arginine reagent butanedione (fig. 8.1) on highly purified Na-K ATPase from rabbit

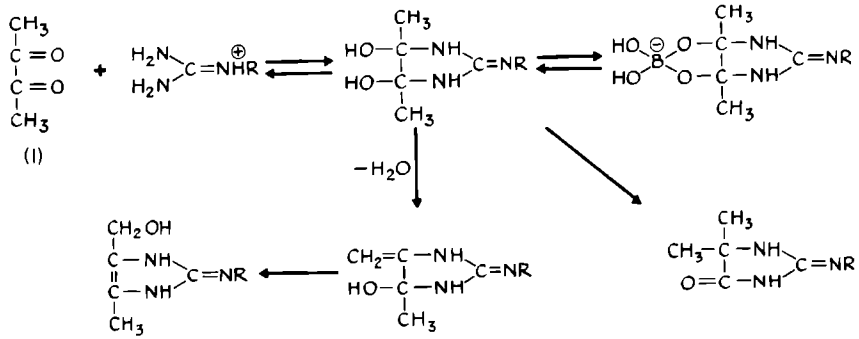


Fig. 8.1 Reaction between butanedione (I) and arginine, as given by Riordan (1973).

kidney outer medulla. We have observed that reaction of Na-K ATPase with butanedione leads to a reversible inhibition of the overall reaction, whereas the K-NPPase reaction is much less inhibited. This suggests that arginyl residues play a specific role in the Na-K ATPase system, probably in the binding of ATP.

Highly purified Na-K ATPase is obtained from rabbit kidney outer medulla microsomes as described in section 3 4. Before modification the enzyme is washed free from Na^+, K^+ and Mg^+ ions and ATP, as described in section 5.2.

Reaction with butanedione is performed in a medium containing 50 (or 125) mM sodium borate buffer (pH 7.5), usually 5 mM $MgCl_2$, 30 µg protein.ml^{-1}, and 2,3 butanedione (Aldrich Europe, Beerse, Belgium) at concentration as stated. After incubation at 25°C samples are removed at the indicated times, and their Na-K ATPase and K-NPPase activities are determined. Since the degree of inhibition differs for various enzyme preparations, a range of concentrations must be used for each new batch in order to determine the sensitivity of the preparation.

Na-K ATPase activity and other enzyme parameters are determined as described in chapter 2.

Reactivation studies are performed after separation of the enzyme from the inhibition medium. Enzyme, which has been approximately 80% inactivated by butanedione is separated from butanedione and buffer by gel-filtration at 4°C over a Sephadex G25 coarse column (0.5 x 10 cm). The column is equilibrated in either 50 mM borate, veronal, hepes or Tris buffer, all at pH 7.5, and the same buffers are used for elution. The Na-K ATPase activity in the second ml eluate is determined immediately after passage through the column and 90 min later. Control samples, which have not been reacted with butanedione, are treated in the same way.

Modification of sulfhydryl groups is carried out by adding 10 µl 10 mM 5,5' dithiobis (2-nitrobenzoic acid) in 30 mM acetate buffer (pH 5.3) to 90 µl of a Na-K ATPase suspension (1 mg/ml) in 125 mM sodium borate buffer (pH 7.5). Butanedione-treated Na-K ATPase has first been chromatographed in borate buffer as described in the preceding paragraph. After 1 hr at 25°C the reaction is complete, and the enzyme is sedimented by centrifugation for 15 min at 10,000 x g. The absorbance is measured spectrophotometrically at 412 nm, using a blank in which 5,5' dithiobis (2-nitrobenzoic acid) has been replaced by the buffer . The number of modified sulfhydryl groups is calculated, using cysteine as a standard.

In some experiments 2 mM butanedione is added to some of the samples after treatment with 5,5' dithiobis (2-nitrobenzoic acid) and the incubation

at 25°C is extended by 30 min. Then dithioerythritol (DTE) (to a final concentration of 10 mM) is added to half of the samples and an equal volume of water to the others. After incubation for another hour at 25°C the Na-K ATPase activity is determined. In some experiments a gel filtration step, as described in the previous paragraph, is applied after DTE treatment and the enzyme assay is carried out in the usual way.

Aminoacid analysis is carried out after heating an enzyme sample in 6 N HCl for 22 hrs at 110°C in a sealed ampule. The enzyme sample consists of butanedione-treated Na-K ATPase, that is concentrated by centrifugation for 30 min at 5000 x g after addition of excess 6 N HCl. Amino acid analysis is carried out in a Rank Hilger Chromaspek amino acid analyzer.

Free aminogroups are determined fluorimetrically using the fluorescamine reagent according to Böhlen et al. (1973) with the exception that the samples are solubilized in 1% SDS (w/v) before adding fluorescamine (Hoffman La Roche, Nutley, N.J. USA).

Protein determinations are performed as described in section 2.9.1.

8.3 Results

8.3.1 Effect on Na-K ATPase activity

Preincubation of purified Na-K ATPase with butanedione in borate buffer leads to inactivation of the enzyme. The degree of inactivation is dependent on a number of factors, such as concentration of butanedione, time of incubation, pH and concentration of the borate buffer and the presence of other ions in the medium.

Fig. 8.2 shows that in the presence of 50 mM borate buffer (pH 7 5) and 5 mM Mg^{2+} the reaction exhibits pseudo first order kinetics up to 90% inactivation at each of the concentrations of butanedione used. The apparent first order rate constant varies proportionally with the reagent concentration, indicating that the reaction obeys second order kinetics. The second order rate constant, which is equal to the apparent first order rate constant divided by the butanedione concentration, amounts to 19 $min^{-1}M^{-1}$ under the conditions of the experiment. The fact that the reaction shows second order kinetics indicates that inactivation of the enzyme is due to the reaction of one residue in the Na-K ATPase complex with one molecule of butanedione. This does not exclude the possibility that more than one vital arginine residue is present in the enzyme, each

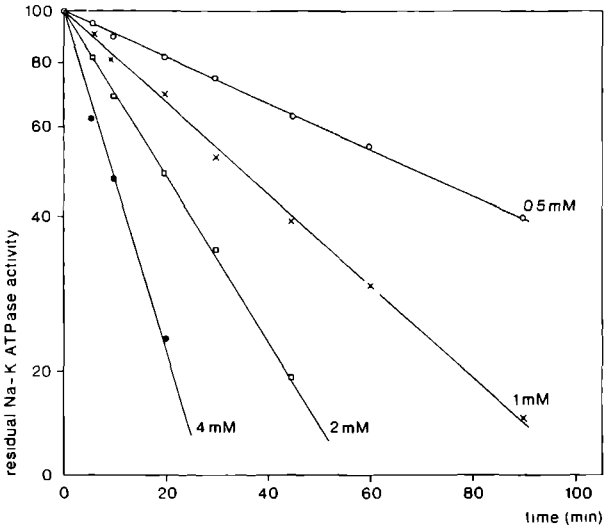


Fig. 8.2 Inactivation by butanedione as a function of time. Na-K ATPase (30 µg/ml) is preincubated at 25°C during the indicated times with various concentrations of butanedione in 125 mM sodium borate buffer, containing 5 mM $MgCl_2$ (pH 7.5). Na-K ATPase is determined as described in section 2.3 (method I). Enzyme activity is expressed as percent of control activity without butanedione.

of which can cause a complete inactivation of the Na-K ATPase activity.

Replacement of the Na^+ ions in the preincubation medium by K^+ leads to a significant reduction in the degree of inactivation. Fig. 8.3 shows that the change in sensitivity towards butanedione already occurs when only a small part of Na^+ is replaced by K^+, a 50% change in sensitivity being found at 0.5 mM K^+ (and 14.5 mM Na^+). Replacement of Mg^{2+} by CDTA has no significant effect on the degree of inhibition by butanedione. However, when the Na-K ATPase preparation has not been washed to remove ATP from the enzyme preparation, a significantly higher degree of inactivation is found in the presence of Mg^{2+} than in that of CDTA. This can be explained by the fact that ATP protects the enzyme against inactivation (see below) and that Mg^{2+} ions remove bound ATP from its binding site.

The pH of the preincubation buffer also has a marked influence on the degree of inactivation. In the pH range of 6.5-8.8 inactivation is most pronounced at pH values above 7.5 (fig. 8.4). Despite the larger inactivation at pH > 8, we have chosen the more physiological pH value of 7.5 for

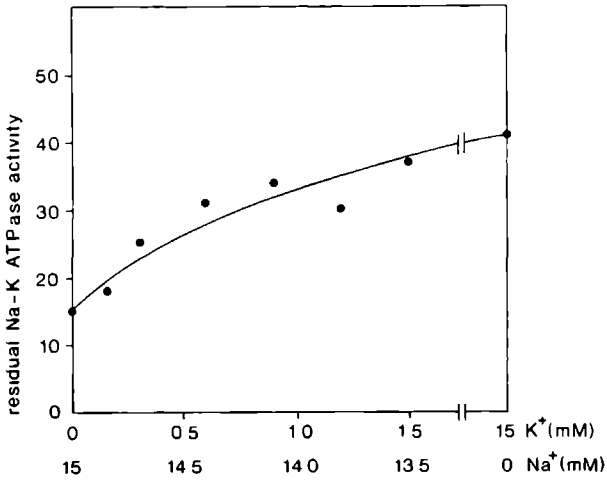


Fig. 8.3 Effect of replacement of Na^+ by K^+ on the inactivation by butanedione. Na-K ATPase (30 µg/ml) is preincubated for 30 min at 25°C with 4 mM butanedione in 50 mM borate buffer (pH 7.5), containing 5 mM Mg^{2+} and concentrations of Na^+ and K^+ as indicated in the figure. Na-K ATPase is determined as described in section 2.3 (method I). Enzyme activity is expressed as percent of control activity without butanedione.

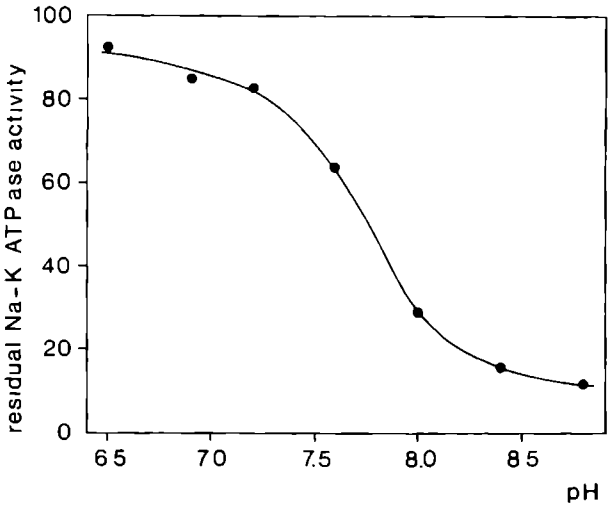


Fig. 8.4 Inactivation by butanedione as a function of pH. Na-K ATPase (30 µg/ml) is preincubated for 30 min at 25°C with 2 mM butanedione in 125 mM sodium borate buffer containing 5 mM $MgCl_2$, previously brought to the indicated pH. Na-K ATPase is determined as described in section 2.3 (method I). Enzyme activity is expressed as percent of control activity without butanedione.

subsequent experiments. The degree of inactivation by butanedione also varies with the borate concentration (fig. 8.5). Maximal inactivation is

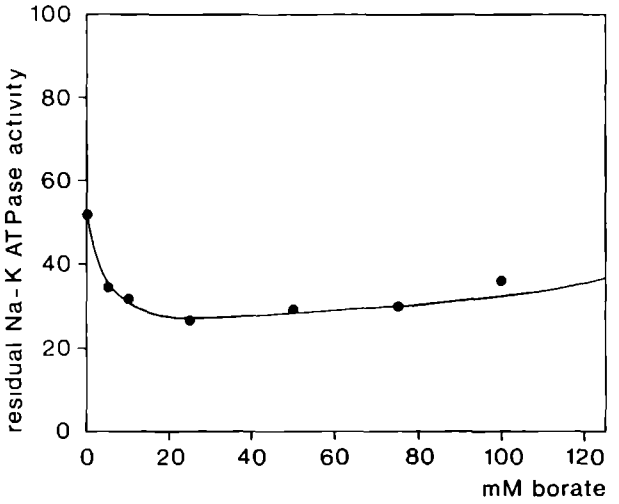


Fig. 8.5 Inactivation by butanedione as a function of borate concentration. Na-K ATPase (30 µg/ml) is preincubated for 30 min at 25°C with 2 mM butanedione in 125 mM buffer, containing 5 mM $MgCl_2$. Mixtures of borate and hepes buffer (pH 7.5) with a total concentration of 125 mM are used. Na-K ATPase is determined as described in section 2.3 (method I). Enzyme activity is expressed as percent of control activity without butanedione.

observed in the presence of 25 mM borate, whereas the effect decreases somewhat at higher borate concentrations. At 200 mM borate the effect of butanedione is markedly reduced. This behaviour agrees qualitatively with that observed for other enzymes (Riordan, 1973; Borders et al., 1975; Bleile et al., 1975).

Reversibility of the inactivation is tested by gel filtration of the inactivated enzyme complex on Sephadex G25, equilibrated with various buffers. The sample eluted with 50 mM borate buffer is not reactivated, whereas the activities of the other samples, which have been eluted with 50 mM veronal, Tris or hepes buffers, are partly restored (Table 8.1). This indicates that the butanedione-enzyme complex is stabilized by borate ions.

The effect of ATP and other nucleotides on inactivation by butanedione

Table 8.1

REVERSIBILITY OF THE INACTIVATION OF Na-K ATPase BY BUTANEDIONE

buffer (50 mM, pH 7.5)	Na-K ATPase activity (percent of control)	
	time after gel filtration	
	0 h	1½ h
borate	15	17
veronal	22	63
hepes	28	88
Tris-HCl	23	65

Na-K ATPase (1.6 mg/ml) is incubated for 30 min at 25°C in 50 mM sodium borate buffer (pH 7.5) containing 5 mM $MgCl_2$ and 5 mM butanedione. Aliquots are filtered through Sephadex G25 columns with the indicated buffer. Na-K ATPase activity is assayed as described in section 2.3 (method I), immediately after gel filtration and 1½ h later. Results are expressed as percent of control activities in the absence of butanedione. Activity before gel filtration is 18% of the control activity.

has been determined. In the presence of Mg^{2+} the inactivation by butanedione is partially prevented by ATP (Fig. 8.6a, upper curve), but the concentration dependence is slight and there is a large variability in the degree of inactivation. When Mg^{2+} is replaced by an equal concentration of CDTA, the protective effect of ATP is more pronounced and is noticable at very low concentrations (fig. 8.6a, lower curve), while the variability in the results is much smaller. ADP also protects against inactivation by butanedione (fig. 8.6b). Of various phosphate compounds applied in low concentrations (10 μM) in the presence of CDTA, only ATP, ADP and to a lesser degree the ATP analogue adenylylimidodiphosphate affect the inactivation by butanedione (Table 8.2). For all three nucleotides the protective effect is much more pronounced in the absence of Mg^{2+}.

8.3.2 Effect on K^+ stimulated p-nitrophenylphosphatase

The K-NPPase activity is much more resistant to inactivation by butanedione than the Na-K ATPase activity. Fig. 8.7 shows the effect of various concentrations of butanedione on the two enzyme activities, measured after preincubation periods of 30 and 120 min at 25°C. This difference in sensitivity towards butanedione is apparent in all experiments.

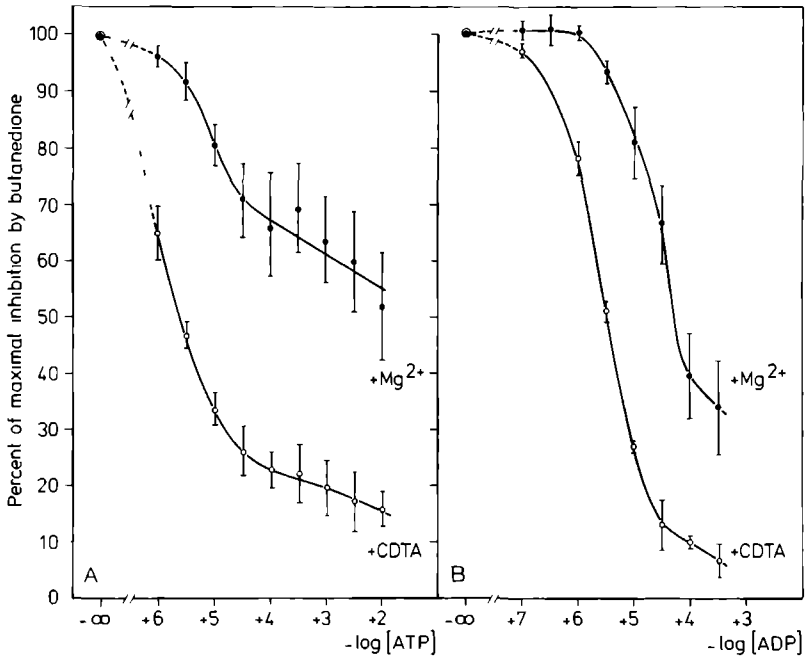


Fig. 8.6 Protective effect of ATP (fig 8 6.A) and ADP (fig 8.6.B) against inactivation by butanedione. Na-K ATPase (30 µg/ml) is preincubated for 30 min at 25°C in 50 mM sodium borate buffer (pH 7.5) containing either 5 mM Mg^{2+} or 5 mM CDTA, 4 mM butanedione and ATP or ADP in the indicated concentrations expressed in mole/l. Na-K ATPase is determined as described in section 2.3 (method I). Results are expressed as percent of inhibition in the absence of added nucleotide, and represent means with S.E.M. of three experiments.

In 22 experiments under various conditions (pH, borate concentration, type and concentration of ligand, butanedione concentration), selected so as to give residual Na-K ATPase activities between 15 and 35% of the butanedione-free control, the residual K-NPPase activity is 75% (S.E.: 2.2) of the control against an average residual Na-K ATPase activity of 25% (S.E.: 1.2). The kinetics for the inactivation of K-NPPase also differ from those for Na-K ATPase, inasfar as no pseudo first-order kinetics are found. These findings suggest that modification of more than one amino acid residue is necessary for complete inactivation of the K-NPPase activity.

The K-NPPase activity can be stimulated by ATP in the presence of

Table 8.2

EFFECT OF PHOSPHATE COMPOUNDS ON INACTIVATION OF Na-K ATPase BY BUTANEDIONE

Substance added (10 µM)	Percent inhibition
-	= 100
ATP	37 ± 5.9
ADP	40 ± 4.5
Adenylyl imido diphosphate	72 + 2.7
5' AMP	102 ± 2.7
3'5' AMP	101 + 2.6
CTP	95 + 2.4
GTP	98 + 3.0
I1P	97 + 2.7
UTP	99 + 1.9
4-nitrophenyl phosphate	98 ± 2.1
Na-orthophosphate	98 ± 3.8

Na-K ATPase (30 µg/ml) is preincubated for 30 min at 25°C n 50 mM sodium borate buffer (pH 7.5) containing 5 mM CDTA, 8 mM butanedione and 10 µM of the indicated compounds. Na-K ATPase determinations are carried out as described in section 2.3 (method I). Results are expressed as percent of the inhibition obtained in the absence of added substance. Results represent means and S.E.M. of three experiments.

Na^+, but only when K^+ is present in suboptimal concentration (section 4.7). We have, therefore, investigated the effect of preincubation with butanedione in the presence of 0.5 mM K^+ with and without ATP. Table 8.3 shows that whereas the K-NPPase activity in the absence of ATP at low K^+ concentration is not reduced by preincubation with butanedione, the stimulating effect of ATP is markedly reduced. This suggests that butanedione reacts with a residue which is involved in the stimulating effect of ATP.

8.3.3 Effects on phosphorylation

Preincubation with butanedione does not only lead to a reduction of the Na-K ATPase and K-NPPase activities, but also to a reduction of the phosphorylation of the enzyme. In table 8.4 is shown that the Na-K ATPase activity and the Na^+ dependent phosphorylation by ATP are inhibited to

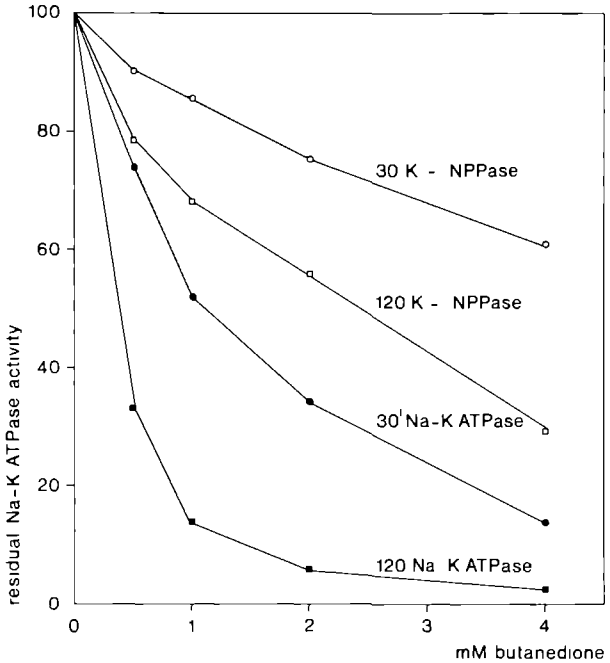


Fig. 8.7 Comparison between inhibition by butanedione of Na-K ATPase and K^+-4-nitrophenylphosphatase activities. Na-K ATPase (30 µg/ml) is pre-incubated for 120 min at 25°C with various concentrations of butanedione in 125 mM borate buffer (pH 7.5), containing 5 mM Mg^{2+}. After 30 and 120 min Na-K ATPase and K^+-4-nitrophenylphosphatase (K-NPPase) activities are determined as described in sections 2.3 (method I) and 2.7. Enzyme activities are expressed as percent of control activity whithout butanedione.

the same extent after reaction with butanedione. The K-NPPase activity and phosphorylation by P_i are inhibited equally, but to a lesser degree than the Na-K ATPase activity.

8.3.4 Effect on sulfhydryl groups

Sulfhydryl residues seem to play an essential role in the active center of Na-K ATPase (chapters 5-7) Notwithstanding earlier findings that butanedione does not affect sulfhydryl groups (Lange et al., 1974; Borders and Riordan, 1975), we have investigated whether the observed effects of butanedione could be due to interference with these groups. The number of reactive sulfhydryl groups before and after treatment with butanedione has been measured by absorbance spectrophotometry at 412 nm

Table 8.3

EFFECTS OF BUTANEDIONE ON K^+ STIMULATED 4-NITROPHENYLPHOSPHATASE ACTIVITY IN THE PRESENCE OF VARIOUS LIGANDS

Ligands present in assay	K-NPPase		ratio
	- butanedione	+ butanedione	
1. normal (5 mM K^+)	100	67 ± 10.1	0.67 ± 0.10
2. 0.5 mM K^+, 20 mM Na^+	22 ± 1.4	20 ± 2.9	0.91 ± 0.08
3. 0.5 mM K^+, 20 mM Na^+, 0.1 mM ATP	55 ± 4	29 ± 3	0.54 ± 0.06
4. Difference 3-2	33 ± 3	9 ± 3	0.27 ± 0.09
	Na-K ATPase		
Ligands present	- butanedione	+ butanedione	ratio
5. normal (10 mM K^+, 110 mM Na^+)	100	19 ± 3	0.19 ± 0.03

Na-K ATPase (31 µg protein.ml^{-1}) is preincubated for 30 min at 25°C in 125 mM sodium borate buffer (pH 7.5) containing 5 mM $MgCl_2$ with and without 2 mM butanedione. The K^+ stimulated 4-nitrophenylphosphatase (K-NPPase) and the Na-K ATPase activities are determined as described in sections 2.3 (method I) and 2.7, with the exception that in the K-NPPase assay media K^+, Na^+ and ATP are present in the concentrations indicated above. In the ouabain containing K-NPPase assay medium K^+ ions are omitted. Results are expressed as percent of the activity measured under optimal conditions for K-NPPase activity, and are given as averages with S.E.M. for 4 experiments.

Table 8.4

EFFECT OF BUTANEDIONE ON PHOSPHORYLATION, Na-K ATPase AND K^+ STIMULATED 4-NITROPHENYLPHOSPHATASE ACTIVITY

	percentage of control
Na^+ dependent phosphorylation by ATP (37°C)	21.8 ± 1.1
Na-K ATPase activity	21.3 ± 1.2
Phosphorylation by P_i	65.0 + 2.5
K-NPPase activity	69.5 ± 3.0

Na-K ATPase (1.6 mg protein.ml^{-1}) is incubated for 30 min at 25°C in 125 mM sodium borate buffer (pH 7.5), containing 5 mM $MgCl_2$ and 5 mM butanedione. The various activities are determined as described in sections 2.3 (method I), 2.5, 2.6 and 2.7. The results are expressed as the percentage of controls to which no butanedione is added (mean of three experiments, with S.E.M.).

after reaction with 5,5' dithiobis (2-nitrobenzoic acid). In both cases the number of titratable sulfhydryl groups was between 9 and 10, suggesting that butanedione does not react with sulfhydryl residues under the conditions of our experiments. However, since the reaction with butanedione is reversible (fig. 8.1) and the one with 5,5' dithiobis (2-nitrobenzoic acid) is irreversible (fig. 6.1), this does not provide absolute proof for the above suggestion.

Hence, we have also studied the interaction of the effects of 5,5' dithiobis (2-nitrobenzoic acid) and butanedione. For this purpose we have made use of the fact that inactivation by butanedione in Tris buffer can be reversed by gel filtration, whereas the effect of 5,5' dithiobis (2-nitrobenzoic acid) can be reversed by the addition of excess dithioerythritol (DTE). If butanedione and 5,5' dithiobis (2-nitrobenzoic acid) do indeed react with different groups on the enzyme, inactivation by one reagent should not influence the effect of the other.

Fig. 8.8 shows the time dependence of the reaction of butanedione with Na-K ATPase, previously treated with 5,5' dithiobis (2-nitrobenzoic acid), compared to that of an untreated control. After reaction with butanedione the samples are treated with DTE before assay of the Na-K ATPase activity. The difference at t=0 is due to incomplete regeneration of the enzyme activity by DTE. The rate of inactivation by butanedione is clearly decreased after pretreatment with 5,5' dithiobis (2-nitrobenzoic acid). Omission of DTE has no effect when butanedione is applied without pretreatment with 5,5' dithiobis (2-nitrobenzoic acid), again suggesting that butanedione does not react with sulfhydryl groups.

In a further experiment we have tested the effect of various combinations of butanedione, 5,5' dithiobis (2-nitrobenzoic acid), DTE and gel filtration on the Na-K ATPase activity. Table 8.5 shows that DTE is only able to reverse the effect of 5,5' dithiobis (2-nitrobenzoic acid), while gel filtration only reverses (partially) the effect of butanedione. When both 5,5' dithiobis (2-nitrobenzoic acid) and butanedione are used, the inactivation can be completely reversed when both DTE and gel filtration are applied.

These experiments strongly suggest that butanedione does not react with sulfhydryl groups in our experiments. The slower reaction with butanedione after pretreatment with 5,5' dithiobis (2-nitrobenzoic acid) is probably due to steric hindrance by the large 5 thio 2-nitrobenzoic acid residue or by a conformational change induced by 5,5' dithiobis (2-nitrobenzoic acid).

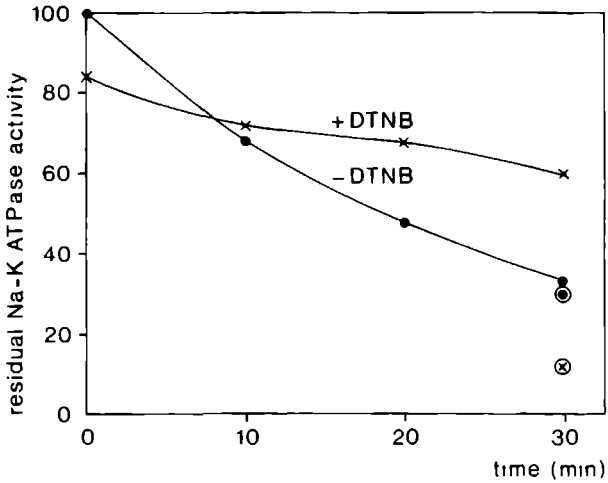


Fig. 8.8 Effect of prior treatment with 5,5' dithiobis (2-nitrobenzoic acid) on inactivation by butanedione. To 600 µl Na-K ATPase (0.1 mg/ml) in 125 mM borate buffer (pH 7.5), containing 5 mM $MgCl_2$, 30 µl of a 4 mM solution of 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) in 30 mM acetate buffer (pH 5.3) or acetate buffer alone is added. After 1 hr preincubation at 25°C butanedione is added (10 mM final concentration) and samples are taken at the indicated times. Dithioerythritol is added to a final concentration of 10 mM. To some samples (⊗, +DTNB; ◉, -DTNB) no dithioerythritol is added. Na-K ATPase activity is determined as described in section 2.3 (method I). Enzyme activities are expressed as percent of control activity without butanedione.

8.3.5 Amino acid analysis

Amino acid analysis after 120 min incubation with 4 mM butanedione does not show a significant decrease in any of the amino acids. Only when excess butanedione is not removed before acid hydrolysis there is a 90% reduction in the arginine content, but this is apparently due to a reaction of butanedione with arginine during acid hydrolysis at 110°C.

Assuming that Na-K ATPase consists of two catalytic subunits, and of two glycoprotein subunits, and has a molecular weight of 250,000 (Kepner and Macey, 1968), the enzyme would contain 80-100 arginine residues per molecule (Kyte, 1972a; Perrone et al., 1975; Hopkins et al., 1976). Since amino acid analysis cannot significantly show a decrease of 5% in the arginine content after reaction with butanedione, this means that less than 5 arginine residues are modified.

Table 8.5

EFFECT OF INTERACTION OF 5,5' DITHIOBIS (2-NITROBENZOIC ACID) AND BUTANEDIONE ON Na-K ATPase ACTIVITY

Treatment				Percent activity
DTNB	Butanedione	DTE	G25	
-	-	-	-	= 100
-	+	-	-	27
+	-	-	-	29
+	+	-	-	13
-	-	+	-	110
-	+	+	-	31
+	-	+	-	97
+	+	+	-	51
-	-	-	+	= 100
-	+	-	+	51
+	-	-	+	24
+	+	-	+	37
-	-	+	+	100
-	+	+	+	58
+	-	+	+	103
+	+	+	+	89

To 190 µl of a Na-K ATPase suspension (1 mg/ml) in 125 mM sodium borate buffer (pH 7.5), containing 5 mM Mg^{2+}, 10 µl of a 4 mM solution of 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) in 30 mM acetate buffer (pH 5.3) or acetate buffer alone is added. The mixture is incubated for 1 hr at 25°C. Then 90 µl aliquots are mixed with 15 µl 11.3 mM butanedione or 15 µl H_2O and incubated for 30 min at 25°C. Of these preparations 10 µl samples are diluted with 190 µl H_2O and 100 µl samples of these dilutions are added to 10 µl of either 100 mM DTE or H_2O and are kept at room temperature for 1 hr. Other samples of the 5,5' dithiobis (2-nitrobenzoic acid) and butanedione-treated preparations and their controls are filtered through small (10 x 0.5 cm) Sephadex G25 columns, or are first treated with DTE for 1 hr followed by gel filtration with 50 mM Tris-HCl. After gel filtration the samples are kept at room temperature for 3 hrs, whereupon Na-K ATPase determinations are carried out as described in section 2.3 (method I). Results are expressed as percent of the appropriate control.

Determination of the number of free amino groups by the fluorescamine method (Böhlen et al., 1973) indicates that after butanedione treatment, leading to 88% inactivation, this number is 99,3% (S.E.: 1 0, 4 experiments) of the control without butanedione. This indicates that there is no significant modification of lysine or lipid aminogroups, in agreement with the findings for the lysine content by amino acid analysis.

8.4 Discussion

This study clearly indicates that Na-K ATPase can be reversibly inactivated by butanedione. This is most likely due to modification of an arginine residue, since it has been shown that this reagent is rather specific. The pH dependance of the reaction (fig. 8.4), the dependence on the borate buffer concentration (fig. 8.5) and the reversibility of the reaction after gel filtration with all buffers except borate (Table 8.2) strongly support this conclusion.

The fact that the reaction obeys second order kinetics indicates that a single amino acid residue is involved in the inactivating effect of butanedione, although modification of additional (non-)essential amino acid residues cannot be excluded. Our inability to find a significant decrease in arginine content by amino acid analysis also indicates that only a few (< 5) arginine residues are modified. In several other cases it has been shown that there are only a few essential arginine residues, and that these are the most reactive ones among all arginines present (Yang and Schwert, 1972; Riordan, 1973; Riordan and Scandurra, 1975; Marcus et al., 1976).

It is unclear why in particular those residues which are important for enzyme activity are the ones that react primarily with butanedione. Powers and Riordan (1975) suggest that residues located in a hydrophobic area would react primarily. It seems to us that such residues would have a lower apparent pK_a value, thus rendering them more reactive towards butanedione. This would also offer an alternative explanation for the pH dependence of the reaction, other than the effects on the borate buffer or another functional group nearby (Lange et al., 1974; Riordan, 1973).

Since in many enzyme modification studies the inactivation reaction can be blocked by the addition of substrates or cofactors, the application of these reagents may be useful in the elucidation of the structure of the

active site. The inactivation of Na-K ATPase can be blocked by ATP, particularly in the presence of CDTA (fig. 8.6), which prevents the formation of a phosphorylated intermediate. The dissociation constant for ATP towards the site, where it protects the enzyme against inactivation by butanedione, is about 1.7 μM, when calculated from the data of fig. 8.6, according to the method of Carter et al. (1968). This value is even smaller than that for the ATP binding that antagonizes inhibition by 5,5' dithiobis (2-nitrobenzoic acid) (5 μM; section 6 4). This suggests that binding to the high affinity AIP binding site (K_{diss} = 0.2 μM) is involved. This suggestion is strongly supported by the fact that ADP and AMPPNP, with about the same affinities for the enzyme as ATP can reduce inactivation by butanedione, when added at a concentration of 10 μM, while other compounds with much lower affinities, like AMP and inorganic phosphate, cannot do this. These results indicate that the arginine residue, which can be modified by butanedione, is located in the high affinity ATP binding center of the enzyme. The positively charged arginine residue may be involved in the binding of the negatively charged part of ATP.

An alternative explanation could be that binding of ATP would induce a change in enzyme conformation, which leads to burying of an essential arginine residue outside the active centre. This alternative seems unlikely to us, since in that case phosphorylation would re-expose the residue. Moreover, the parallelism between our findings and the fact that arginine is involved in the binding of negatively charged substrates and cofactors in many other enzymes (Riordan et al., 1977) seems more than accidental to us.

The much smaller protection by ATP in the presence of Mg^{2+} ions (combined with Na^+ ions from the buffer) is probably due to phosphorylation of the enzyme, and subsequent release of ADP (Karlish et al., 1976). It is known that the presence of Mg^{2+} ions reduces the affinity of the enzyme for ADP (Kanilke et al., 1973). This mechanism also explains the difference in protecting effects of ADP in the presence or absence of Mg^{2+} ions.

The reduced inactivation upon partial replacement of Na^+ ions by K^+ ions seems to be specific for Na-K ATPase, since it has not been reported for the inactivation of other enzymes by butanedione. This suggests that the presence of Na^+ ions in the preincubation medium makes the specific arginine residues more accessible to butanedione, probably due to a change

in conformation of the enzyme. Nørby and Jensen (1971) and Hegyvary and Post (1971) have observed that the affinity of the enzyme for ATP is also lowered by addition of K^+ ions. This may mean that a conformational change of the ATP binding site, induced by K^+ ions leads to a reduction in the binding of ATP as well as to a reduction in the accessibility of the essential arginine residue towards butanedione.

Modification of sulfhydrylgroups also leads to an inactivation of the Na-K ATPase activity. This inactivation can also be prevented by the addition of ATP in the absence of Mg^{2+} ions, apparently by binding of ATP to the high affinity binding site (section 6.4). Patzelt-Wenczler et al. (1975) suggest that a sulfhydryl group in the ATP binding center is involved in the binding of ATP via the 6 aminogroup. Since binding of ATP to a positively charged arginine residue probably involves a negatively charged phosphate group, these results do not necessarily conflict. On the contrary, they may well explain the fact that modification of sulfhydryl residues with 5,5' dithiobis (2-nitrobenzoic acid) prevents reaction with butanedione (fig. 8.8, table 8.5). However, other explanations, like an aspecific conformational change due to the prior reaction with 5,5'dithiobis (2-nitrobenzoic acid) cannot be excluded.

The equal sensitivity of the Na^+ dependent phosphorylation and the Na-K ATPase activity towards butanedione can be explained by assuming that the modification with butanedione prevents binding of ATP to the enzyme, and thus all reactions that require this step. The lower sensitivity of the K-NPPase activity and the phosphorylation by P_i (both equal in sensitivity) can be explained by assuming that the binding of NPP and P_i takes place on a site, different from the site for ATP binding.

Several reaction mechanisms have been suggested, in which two reactive centers exist on the enzyme, both located on different catalytic subunits. Robinson (1976) suggests that a high affinity ATP binding center would catalyze the phosphorylation by ATP, whereas a low affinity center would be involved in the K-NPPase reaction. In addition, Jørgensen (1977) suggest that the phosphorylated high affinity center serves as a substrate for the phosphatase activity (a kinase vs. a phosphatase activity). The Na-K ATPase reaction mechanism at physiological substrate conditions would then require both active centers, whereas the K-NPPase activity would only require the phosphatase site. The different sensitivities of the Na-K ATPase

and K-NPPase activities to butanedione could then be explained by assuming that reaction with butanedione primarily prevents binding of ATP to the high affinity site, and thus phosphorylation of this site. This would also explain why the stimulation by ATP of the K-NPPase activity is inhibited, since phosphorylation of the high affinity site of this enzyme would be involved in this activation (see section 2.7).

Our conclusion that an arginine residue plays a role in the binding of ATP to Na-K ATPase is in agreement with the findings for several other enzyme systems, viz. Ca,Mg ATPase (Murphy, 1976b), mitochondrial ATPase (Marcus et al., 1976), creatine kinase (Borders and Riordan, 1975), glutamine synthetase and carbamoyl phosphate synthetase (Powers and Riordan, 1975). Moreover, Borders and Riordan (1975) have preliminary evidence that butanedione inhibits four other kinases, in which an ATP binding site may be involved. Peculiar to Na-K ATPase is that it requires only 1/100th of the ATP concentration needed by the other enzymes to prevent inactivation by butanedione, indicating a much higher affinity of Na-K ATPase for ATP.

CHAPTER 9

EFFECTS OF CROSSLINKING ON THE ENZYME

9.1 Introduction

Crosslinking of proteins with bifunctional reagents can be used to determine the number of subunits and their spatial localization in complex enzymes (Hajdu et al., 1976; Peters and Richards, 1977). The use of this approach seems indicated, since there is still uncertainty about the exact number of subunits that form the active enzyme complex of Na-K ATPase. There are two different subunits, a 100,000 and a 50,000 subunit. The larger one is the catalytic (α) subunit, of which there appear to be two present per molecule (section 1.7). Uncertain is still whether there are one or two of the 50,000 MW glycoprotein (β) subunits present per molecule. The aim of this part of our study is to discriminate between these two possibilities by the sequential use of two crosslinking reagents. One of these reagents can only react with sulfhydryl groups, and will therefore crosslink only the catalytic subunits (see section 7.3.1). As reagents for this crosslinking reaction two bifunctional sulfhydryl reagents (bis maleimidomethyl ether and p-azophenyl N,N' dimaleimide) and cupric (o-phenanthroline)$_2$ sulfate have been used. The other reagent, N,N' dimethyl-suberimidate, will crosslink the catalytic subunit with the glycoprotein subunit (Kyte, 1972a) Determination of the molecular weights of the resulting aggregates by means of SDS polyacrylamide gel electrophoresis should then allow to distinguish between the $\alpha_2\beta_2$ and $\alpha_2\beta$ configurations.

Unfortunately, crosslink studies of Na-K ATPase very easily lead to artifacts, and so it has not been possible to draw definite conclusions about the subunit composition of the enzyme complex.

9.2 Materials and Methods

Purified Na-K ATPase is obtained from rabbit kidney outer medulla as described in section 3.4. Before crosslinking, the enzyme is washed free from Na^+, K^+ and Mg^{2+} ions and ATP, as described in section 5.2.

Reaction with cupric (o-phenanthroline)$_2$ sulfate is performed in 25 mM Tris-HCl (pH 7.6), 0.1 mM $CuSO_4$, 0.2 mM o-phenanthroline, 1.5 mg protein. $.ml^{-1}$ and, where indicated, 10 mM Tris ATP. The reaction, either at 37°C or 0°C is stopped at the stated time by 30-fold dilution of a 30 µl aliquot of the reaction mixture in 50 mM Tris-HCl (pH 7.6) and 1 mM CDTA. Residual Na-K ATPase activity is determined in an aliquot of the stopped reaction mixture and the remainder is used for protein composition analysis on SDS polyacrylamide gels.

Reaction with bis maleimidomethyl ether (Bis) or p-azophenyl N,N' dimaleimide (Azo-bis) is carried out in a medium containing: 25 mM imidazole-HCl (pH 7.5), 4 mM CDTA, 0.75 mg protein.ml^{-1}, 5% (v/v) N,N dimethylformamide, and where stated 10 mM Tris ATP and either Bis of Azo-bis at the indicated concentrations. After incubation for 30 min at either 37°C or 0°C, residual Na-K ATPase activity is determined. Protein composition of the samples (70 µg protein) is determined by SDS polyacrylamide gel electrophoresis. Because of their low solubility in water and the slow dissolving rate, the two reagents are added to the incubation medium as a concentrated solution in N,N dimethylformamide. A control experiment is carried out to correct for Na-K ATPase inhibition by N,N dimethylformamide.

Reaction with N,N' dimethylsuberimidate is performed in a medium containing: 0.1 M triethanolamine-HCl (pH 10.0), various amounts of N,N' dimethylsuberimidate (varying from 0.33-1.0 mg.ml^{-1}) and 1 mg protein.ml^{-1}, for 30 min at 25°C. After this period the reaction mixture is stored at 0°C. Subsequent treatment with Bis or Azo-bis is performed by addition of Bis or Azo-bis, dissolved in N,N dimethylformamide at the stated concentration to the reaction mixture and subsequent incubation for 30 min at either 37°C or 0°C. The final N,N dimethylformamide concentration in the incubation mixture is then 5% (v/v).

Gel electrophoresis is carried out on SDS polyacrylamide gels according to Davies and Stark (see section 2.9.2)

The Na-K ATPase activity is determined as described in section 2.3 (method I).

Crosslinking reagents: o-phenanthroline (1,10 phenanthroline) is obtained from Merck (Darmstadt, W-Germany), p-azophenyl N,N' dimaleimide is a gift of Dr. C. Regan, who synthetized it as described by Fasold et al. (1963). Bis maleimidomethyl ether is synthetized according to Tawney et al.

(1961). N,N' dimethylsuberimidate is obtained from Pierce (Rockford, USA).

9.3 Results

9.3.1 Analysis of crosslink products on gel electrophoresis

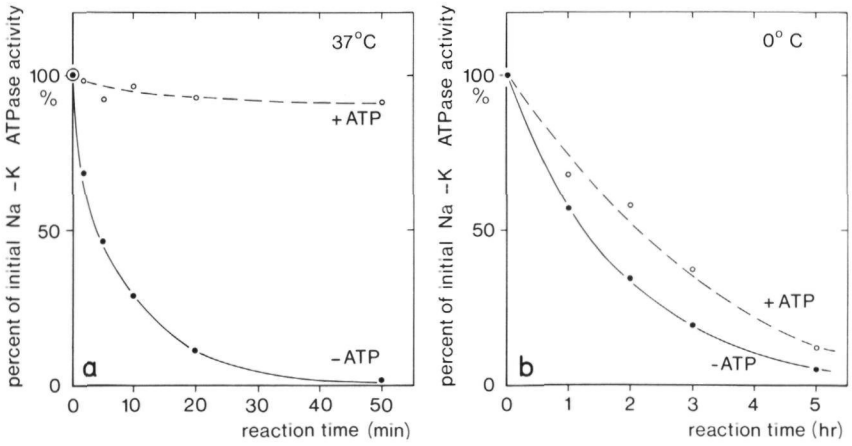
The occurrence of crosslinking is investigated by means of analytical SDS polyacrylamide gel electrophoresis in 3.5% (total acrylamide) gels, according to Davies and Stark (see section 2.9.2). This system has the advantage over the Laemmli gel system that proteins with molecular weights over 150,000 are separated. The disadvantage of this sytem is that the mobility of the glycoprotein sugunit is decreased, so that for the glycoprotein subunit an apparent molecular weight of 80,000 is found, which is very near to that of the catalytic subunit (95,000) (see also fig. 4.2).

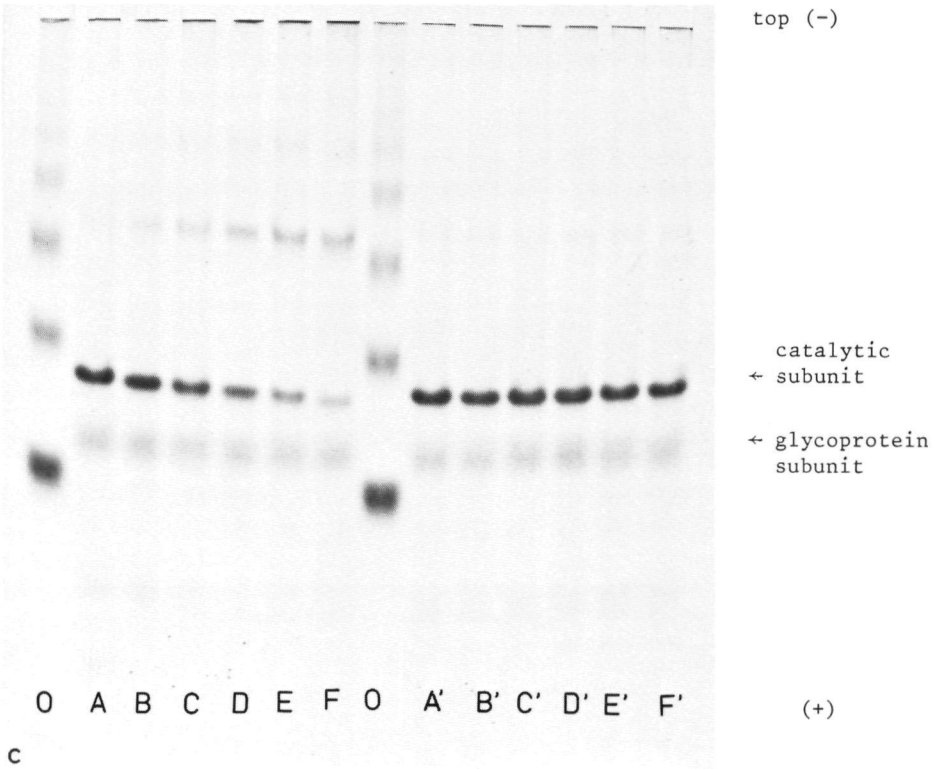
Thiols, normally present during solubilization of the proteins in SDS, will reduce disulfide bridges, including those formed by reaction in presence of cupric (o-phenanthroline)$_2$ sulfate. Therefore N-ethylmaleimide is added to the solubilization mixture in order to prevent crosslinking due to disulfide formation by air oxidation. An advantage of alkylation of sulfhydryl groups by N-ethylmaleimide is that for unknown reasons the mobility of the catalytic subunit is decreased, which results in better separation from the glycoprotein subunit.

The better separation of proteins with molecular weights over 150,000 in 3.5% polyacrylamide gels means that a protein band with an apparent molecular weight of twice that of the catalytic subunit is observed upon gel electrophoresis of a pure Na-K ATPase preparation. Since this probably represents a dimer of the catalytic subunit (Sweadner, 1977), it becomes very difficult to observe the formation of a crosslink between two catalytic subunits. Therefore, a good criterion for the formation of a crosslink is the disappearance of the parent protein band(s) simultaneously with the appearance of the polymers.

9.3.2 Effects of incubation with cupric (o-phenanthroline)$_2$ sulfate

Fig. 9.1 shows that incubation of Na-K ATPase with cupric (o-phenanthroline)$_2$ sulfate (fig. 9.2) inhibits the enzyme activity. At 37^{o}C the inhibition is completed notably faster than at 0^{o}C (50 min vs. > 5 hrs). The inhibition at 37^{o}C is antagonized very effectively by the addition of


percent of initial Na -K ATPase activity
100 %
50
0
37°C
+ATP
-ATP
a
0 10 20 30 40 50
reaction time (min)
0° C
+ATP
-ATP
b
0 1 2 3 4 5
reaction time (hr)


top (-)
catalytic
← subunit
← glycoprotein
subunit
O A B C D E F O A' B' C' D' E' F'
(+)
c

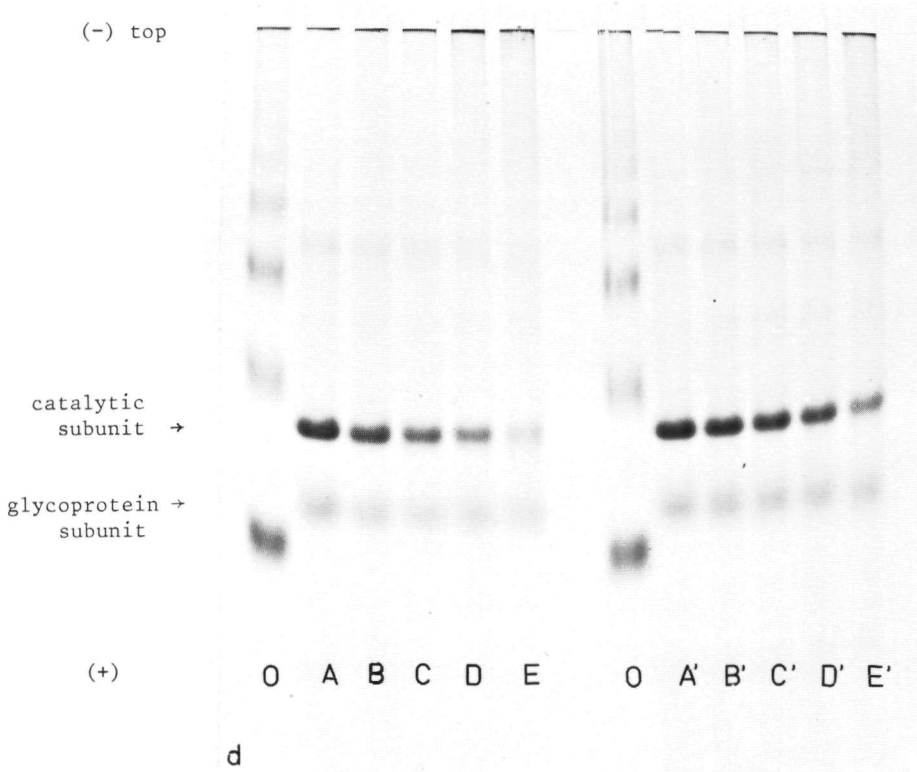


Fig. 9.1 Effects of incubation with cupric o-phenanthroline. The incubation of purified Na-K ATPase from rabbit kidney outer medulla with cupric $(o\text{-phenanthroline})_2$ sulfate (CuP) is performed in a medium containing 25 mM Tris-HCl (pH 7.6), 0.1 mM $CuSO_4$, 0.2 mM o-phenanthroline, 1.5 mg protein. $.ml^{-1}$, and 0 or 10 mM Tris ATP. The reaction, either at 37°C or at 0°C is ended at the stated time by 30-fold dilution with 50 mM Tris-HCl (pH 7.5), 1 mM CDTA.

a. Residual Na-K ATPase activity after incubation at 37°C in presence of 0 (—•—) or 10 mM (---o---) Tris ATP.
b. Residual Na-K ATPase activity after incubation at 0°C in the presence of 0 (—•—) or 10 mM (---o---) Tris ATP.
c. SDS electropherogram of the reaction products after incubation of Na-K ATPase with CuP at 37°C for 0 min (A), 1 min (B), 5 min (C), 10 min (D), 20 min (E) and 50 min (F). The presence of ATP in the reaction mixture is indicated by an apostrophe ('). O represents a calibration mixture of bovine serum albumine and its polymers.
d. SDS electropherogram of the reaction products after incubation of Na-K ATPase with CuP for 0 hr (A), 1 hr (B), 2 hr (C), 3 hr (D) and 5 hr (E). The presence of ATP in the reaction mixture is indicated by an apostrophe ('). O represents a calibration mixture of bovine serum albumine and its polymers.

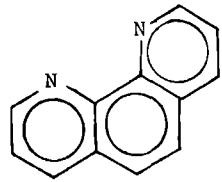


Fig. 9.2 Structure of 1,10 phenanthroline

ATP (fig. 9.1a). ATP also seems to have an antagonizing effect on the inhibition at $0^{\circ}C$, but to a lesser degree (fig. 9.1b).

Treatment of Na-K ATPase with cupric (o-phenanthroline)$_2$ sulfate at $37^{\circ}C$ causes crosslinking of the catalytic subunits (fig. 9.1c) when the enzyme activity is also inhibited (i.e. after reaction in the absence of ATP). No crosslink products are formed at $0^{\circ}C$ (fig. 9.1d) although the enzyme activity is inhibited.

9.3.3 Effects of reaction with bis maleimidomethyl ether

Treatment of purified Na-K ATPase, prepared from rabbit kidney outer medulla with Bis (fig. 9.3) results in inactivation of the enzyme. The

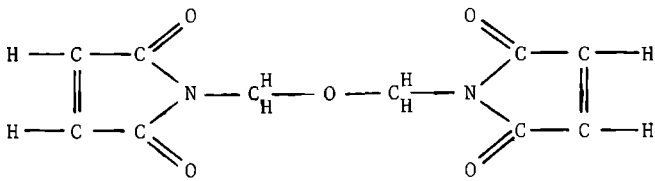


Fig. 9.3 Structure of bis-maleimidomethyl ether.

degree of inhibition depends on time of reaction, reaction temperature, additives in the reaction medium and concentration of Bis. In fig. 9.4a and b the inhibition of Na-K ATPase activity after treatment with Bis under four different conditions is shown. It is clear that the Na-K ATPase activity is inhibited after reaction with various concentrations of Bis at $37^{\circ}C$. The concentration of Bis resulting in 50% inhibition is about 8 µM

(pI_{50} ~ 5.1). The inhibition reaction does not follow pseudo first order kinetics and is complete within 10 minutes (data not shown). At 0°C the reactivity is much less and the concentration of Bis resulting in 50% inhibition is about 10-fold greater. Addition of 10 mM Tris ATP to the reaction media antagonizes the inhibition of the Na-K ATPase activity by Bis at 37°C as well as at 0°C (fig 9.4a and b). In figs. 9.4c and d the protein distribution patterns of Na-K ATPase preparations after inhibition by reaction with Bis are shown on SDS electropherograms. After reaction with Bis the catalytic subunit is polymerized when the enzyme activity is inhibited (fig. 9.4c and fig. 9.4a). After reaction of the enzyme with Bis in the presence of ATP, no crosslink is formed, but this is possibly due to the lesser inhibition of the enzyme activity. After reaction between enzyme and Bis at 0°C no crosslink can be observed (fig. 9.4c) whether the enzyme activity is inhibited or not. ATP has no effect on crosslinking under these circumstances.

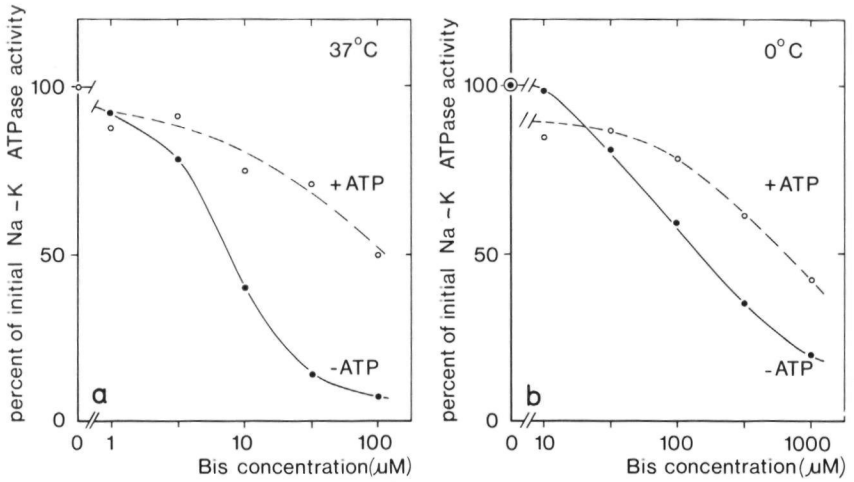
9.3.4 Effects of reaction with p-azophenyl N,N' dimaleimide

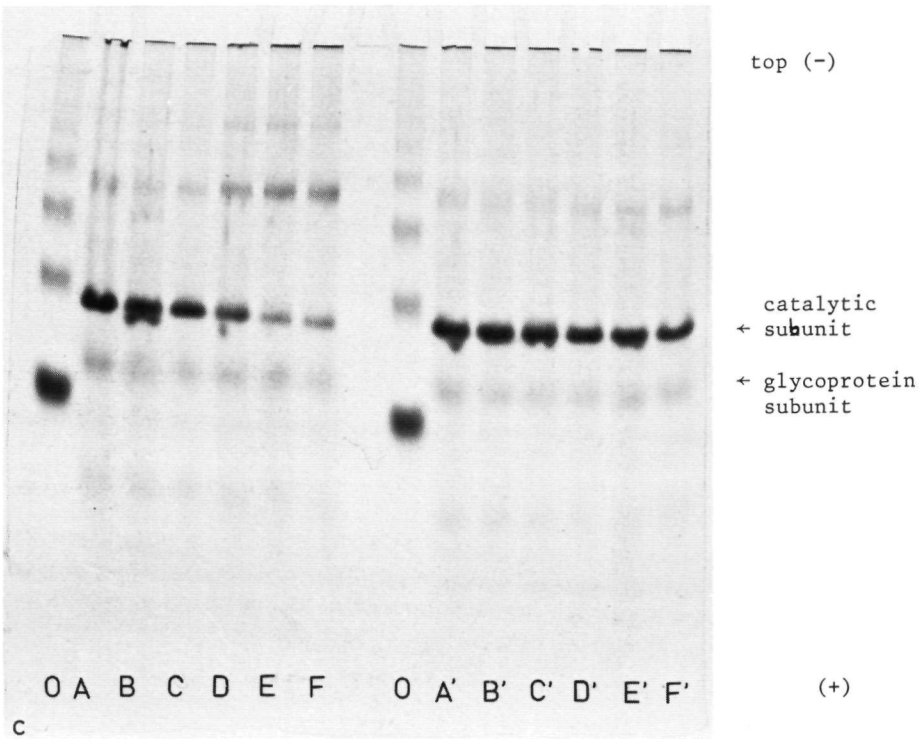
Treatment of Na-K ATPase with Azo-bis (fig. 9.5) results in inhibition of the enzyme activity (fig. 9.6a and b). At 37°C the final inhibition percentage depends on the Azo-bis concentration in the reaction medium, 50% inhibition being obtained at 9.8 µM Azo-bis in the medium (pI_{50} 5.0). At 0°C the inhibition seems to be independent of the reagent concentration (fig. 9.6b). Addition of ATP to the reaction medium does not affect the inhibition at 37°C as well as at 0°C.

Reaction of Na-K ATPase with Azo-bis results in polymerization of the catalytic subunit, apparently in parallel with the inhibition of the enzyme activity. This is observed under all reaction conditions (fig. 9.6c and d).

9.3.5 Effects of sequential reaction with N,N' dimethylsuberimidate and p-azophenyl N,N' dimaleimide

Fig. 9.7 shows that after reaction of purified Na-K ATPase with various amounts of N,N' dimethylsuberimidate (fig. 9.8) a polymer is formed with an apparent molecular weight of 180,000. This would seem to be the product of a crosslink between a glycoprotein and a catalytic subunit. Also polymers with apparent molecular weights of 310,000 and


37°C
0°C
+ATP
-ATP
percent of initial Na-K ATPase activity
Bis concentration(μM)
a
b
0
50
100
0 1 10 100
0 10 100 1000


top (-)
catalytic subunit
glycoprotein subunit
0 A B C D E F
0 A' B' C' D' E' F'
(+)
c

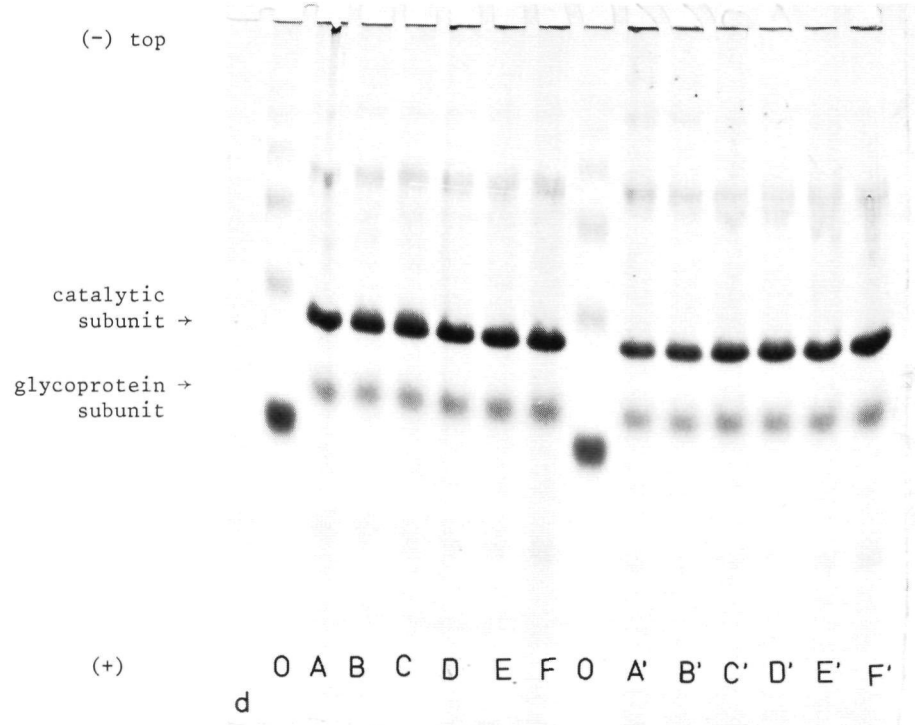


Fig. 9.4 Effects of bis maleimidomethyl ether on Na-K ATPase. The reaction between purified Na-K ATPase from rabbit kidney outer medulla and bis maleimidomethyl ether (Bis) is carried out in a medium containing 25 mM imidazole-HCl (pH 7.5), 4 mM CDTA, 0.75 mg protein.ml^{-1}, 5% (v/v) N,N dimethylformamide, Bis at the stated concentrations, and 0 or 10 mM Tris ATP.

a. Residual Na-K ATPase activity after incubation for 30 min at 37°C in the presence of 0 (—•—) or 10 (---o---) mM Tris ATP.
b. Residual Na-K ATPase activity after incubation for 30 min at 0°C in the presence of 0 (—•—) or 10 (---o---) mM Tris ATP.
c. SDS electropherogram (3.5% polyacrylamide) of the reaction products after reaction at 37°C between Na-K ATPase and Bis. The Bis concentration during reaction amounts to 0 µM (A), 1 µM (B), 3 µM (C), 10 µM (D), 30 µM(E) and 100 µM (F). The presence of ATP in the reaction mixture is indicated by an apostrophe ('). O shows a calibration mixture of bovine serum albumine and its polymers.
d. SDS electropherogram (3.5% polyacrylamide) of the reaction products after reaction at 0°C. The Bis concentration during reaction amounts to 0 µM (A), 10 µM (B), 30 µM (C), 100 µM (D), 300 µM (E) and 1000 µM (F). The presence of ATP in the reaction mixture is indicated by an apostrophe ('). O shows a calibration mixture of bovine serum albumine and its polymers.

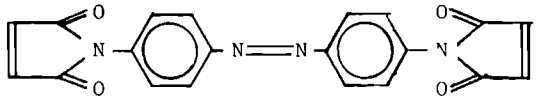


Fig. 9.5 Structure of p-azophenyl N,N' dimaleimide.

360,000 are formed. The 310,000 protein band could represent a crosslink between a preformed dimer of the catalytic subunit and a glycoprotein subunit. The faint 360,000 protein band could either be a crosslink of two catalytic and two glycoprotein subunits or a trimer of the catalytic subunit. Favouring the latter explanation is the observation that the trimer of the catalytic subunit, observed after treatment with Azo-bis, has an identical mobility. As already shown in fig. 9.5c, reaction between Na-K ATPase and Azo-bis results in polymerization of the catalytic subunit, a dimers/trimer and even a tetramer being observed.

Sequential treatment with N,N' dimethylsuberimidate and Azo-bis results in the formation of various crosslink products. The following protein bands can be observed. 80,000 (glycoprotein subunit), 120,000 (catalytic subunit), 180,000 (glycoprotein + catalytic subunit), 240,000 (dimer of catalytic subunits), 310,000 (2 catalytic subunits + 1 glycoprotein subunit), 360,000 (trimer of catalytic subunit, or 2 catalytic + 2 glycoprotein subunits) and 420,000 (crosslink product of unknown compostion). After sequential treatment with N,N' dimethylsuberimidate and Bis (the latter reaction at 37°C) and identical protein distribution pattern is observed (result not shown).

9.4 Discussion

Three crosslinking sulfhydryl reagents have been used, which have some characteristic differences. Cupric (o-phenanthroline)$_2$ sulfate catalyzes the air oxidation of sulfhydryl groups of the protein, resulting in the formation of disulfide bridges. This means that the two reacting groups must be located close to each other (1 to 2 Å). Bis maleimidomethyl ether and p-azophenyl N,N' dimaleimide are bifunctional reagents which react with sulfhydryl groups like N-ethylmaleimide does (cf. fig. 5.1) They are hydrophobic compounds. They differ in the lengths of the crosslinks, which are formed. Bis will span distances of up to 12 Å, whereas Azo-bis will span distances of 22 Å. Another difference is that in Bis the maleimide

moieties are allowed to twist and rotate around the ether bond. In azo-bis these motions will be more restricted because of the large number of double bonds, so that the reagent will behave more like a rod.

Reaction of the enzyme with one of these crosslinking sulfhydryl reagents leads to inhibition of the Na-K ATPase activity. The inhibition is antagonized in some cases by the presence of 10 mM ATP in the reaction medium (under non-phosphorylating conditions). Inhibition of the Na-K ATPase activity is not always accompanied by crosslink formation. A striking difference between the inhibition by the bifunctional reagents and the monofunctional N-ethylmaleimide is the half-maximal inhibitory concentration, which at 37°C is far lower for the crosslinking reagents. 8-10 μM versus 700 μM for N-ethylmaleimide.

In table 9.1 the results obtained with the three crosslinking sulfhydryl reagents are summarized. Only crosslinks between catalytic subunits

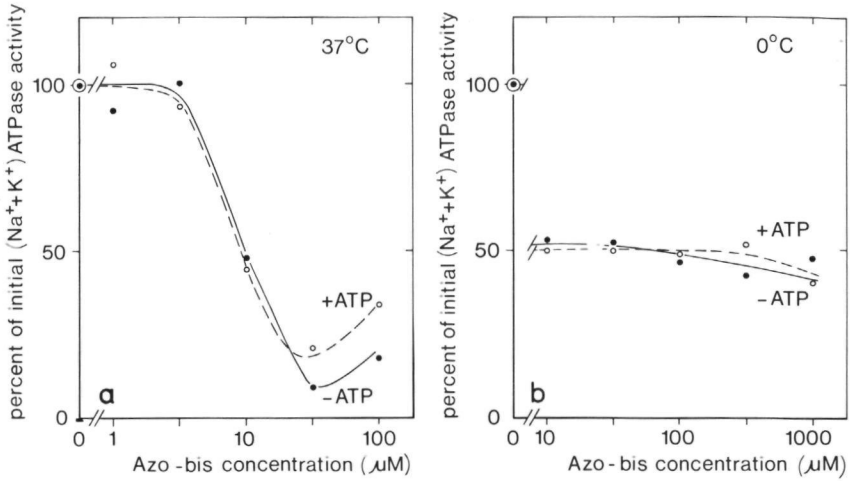
Table 9.1

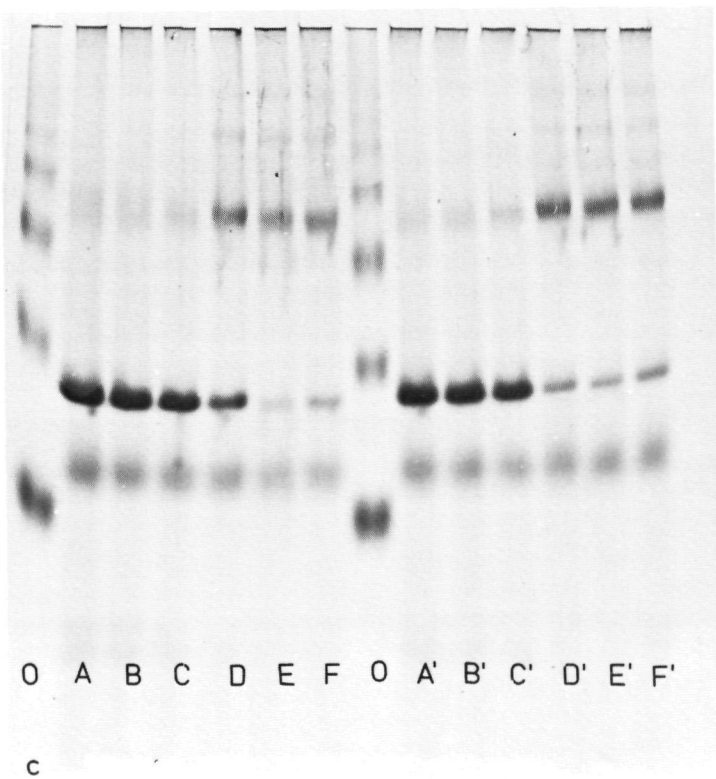
EFFECTS OF CROSSLINKING AGENTS ON Na-K ATPase UNDER VARIOUS CONDITIONS

Agent	Reaction temp.	ATP added (10 mM)	Inhibition of enzyme activity	Crosslinking observed
Bis	37°C	-	+	+
		+	+*	-
	0°C	-	+	-
		+	+*	-
Azo-bis	37°C	-	+	+
		+	+	+
	0°C	-	+	+
		+	+	+
CuP	37°C	-	+	+
		+	-	-
	0°C	-	+	-
		+	+*	-

The crosslinked products are polymers of 100,000 subunits. *Slight protective effects of ATP are observed.

are observed. This is probably due to the fact that nearly all sulfhydryl groups are located on the catalytic subunits (see section 7.3.1) Protective


37°C
0°C
percent of initial (Na+ + K+) ATPase activity
100
50
0
+ATP
-ATP
a
b
0 1 10 100
0 10 100 1000
Azo-bis concentration (μM)


top (-)
catalytic subunit
glycoprotein subunit
O A B C D E F O A' B' C' D' E' F'
(+)
c

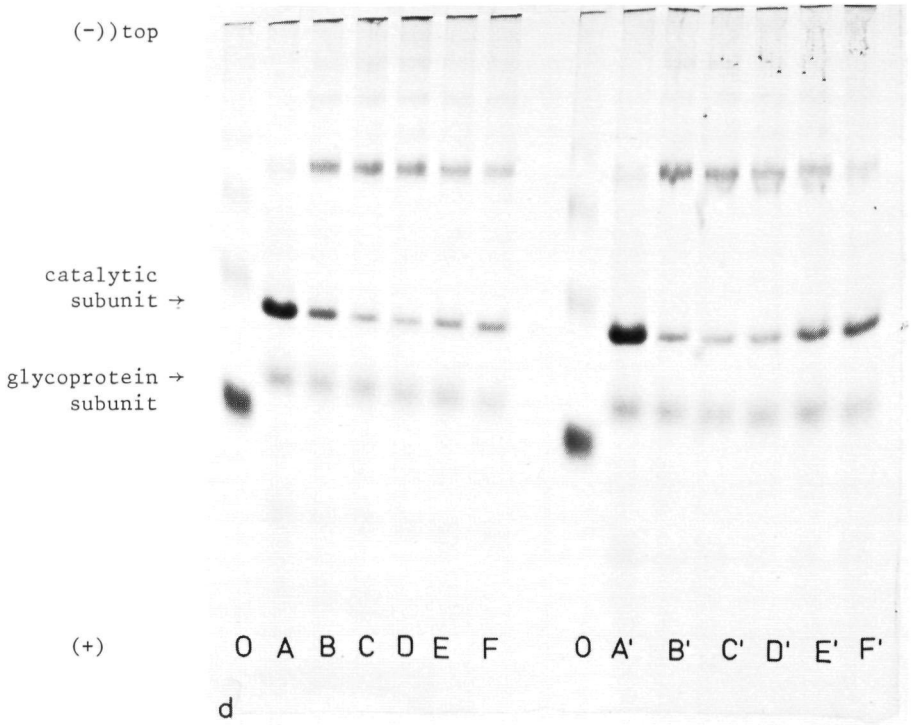


Fig. 9.6 Effects of reaction with p-azophenyl N,N' dimaleimide. The reaction between purified Na-K ATPase from rabbit kidney outer medulla and p-azophenyl N,N' dimaleimide (Azo-bis) is carried in a medium containing 25 mM imidazole-HCl (pH 7.5), 4 mM CDTA, 0.75 mg protein.ml^{-1}, 5% (v/v) N,N dimethylformamide, Azo-bis at the stated concentrations and 0 or 10 mM Tris ATP.

a. Residual Na-K ATPase activity after incubation for 30 min at 37°C in the presence of 0 (—•—) or 10 (---o---) mM Tris ATP.

b. Residual Na-K ATPase activity after incubation for 30 min at 0°C in the presence of 0 (—•—) or 10 (---o---) mM Tris ATP.

c. SDS electropherogram (3.5% polyacrylamide) of the reaction products after reaction at 37°C between Na-K ATPase and Azo-bis. The Azo-bis concentration during reaction amounts to 0 µM (A), 1 µM (B), 3 µM (C), 10 µM (D), 30 µM (E) and 100 µM (F). The presence of ATP in the reaction mixture is indicated by an apostrophe ('). O shows a calibration mixture of bovine serumalbumine and its polymers.

d. SDS electropherogram (3.5% polyacrylamide) of the reaction products after reaction between Na-K ATPase and Azo-bis at 0°C. The Azo-bis concentration during reaction amounts to 0 µM (A), 10 µM (B), 30 µM (C), 100 µM (D), 300 µM (E) and 1000 µM (F). The presence of ATP in the reaction mixture is indicated by an apostrophe ('). O shows a calibration mixture of bovine serumalbumine and its polymers.

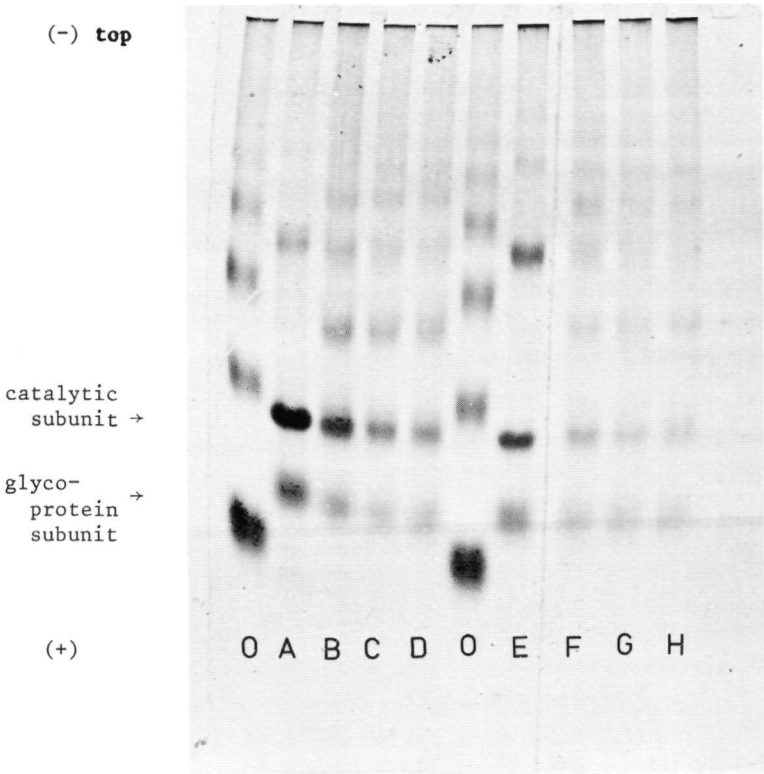


Fig. 9.7 Effects of sequential reaction with N,N' dimethylsuberimidate and p-azophenyl bis maleimide. The reaction between N,N' dimethyl suberimidate (DSI) and purified Na-K ATPase from rabbit kidney outer medulla is performed in a medium containing 0.1 M triethanolamine-HCl (pH 10.0), 1 mg protein.ml^{-1} and N,N' dimethylsuberimidate at the stated concentration for 30 min at 25°C. Subsequent treatment with p-azophenyl N,N' bis maleimide (Azo-bis) is performed by addition of Azo-bis to a final concentration of 0 or 1 mM and by incubation for 30 min at 0°C. SDS electropherograms (3.5% polyacrylamide) of reaction products after reaction with DSI alone (A, B, C and D respectively) and after subsequent treatment with Azo-bis (E, F, G and H) are shown.

	Reaction conditions	
	DSI concentration (mg.ml^{-1})	Azo-bis concentration (mM)
A	0	0
B	0.33	0
C	0.66	0
D	1.0	0
E	0	1
F	0.33	1
G	0.66	1
H	1.0	1

O represents a calibration mixture of bovine serum albumine and its polymers.

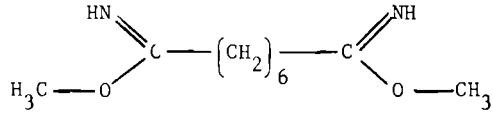


Fig. 9.8 Structure of N,N' dimethylsuberimidate (DSI).

effects of ATP on the inhibition may be due to shielding of an essential sulfhydrylgroup or to a conformational change of the enzyme leading to a change in distance between the two sulfhydryl groups. The effect of ATP on the CuP catalyzed crosslinking may also be caused by complexation of Cu^{2+} ions with ATP.

Inhibition of the Na-K ATPase activity by the crosslinking agents could be due to the following effects.

a. Reaction with a group essential for enzyme activity;
b. Intramolecular crosslinking within the same subunit;
c Intramolecular crosslinking between two subunits within the same enzyme complex;
d Intermolecular crosslinking between subunits of neighbouring enzyme molecules.

Inhibition through one of these three types of crosslinking (b, c, d) could be due to involvement of an essential sulfhydrylgroup in the cross-link formation, or to the hindering of conformational changes occurring during enzyme activity. It will be very difficult to determine the exact mechanism operating in each case; in most cases combined effects will take place. Obviously, only crosslinks formed in reactions of categories c and d will be detected in SDS electropherograms.

It is very difficult to discriminate between intra- and intermolecular crosslinks The chance for an intermolecular crosslink reaction will increase with the protein concentration. Since Na-K ATPase is a membrane bound protein, the local concentration of the enzyme will be rather high and cannot be lowered by dilution of the enzyme in the reaction mixture. At elevated temperatures the membrane fluidity and thus the lateral mobility of the enzyme molecules are increased, and hence the frequency of collisions between these molecules will rise. This means that the length of the crosslinking reagent will not guarantee an intramolecular cross-link. At lower reaction temperature membrane fluidity and collision rate

will decrease, which will also decrease the chance of intermolecular crosslinking.

Winter and Liang (1977) and Winter et al. (1977) report that after incubation of Na-K ATPase with cupric (o-phenanthrolin)$_2$ sulfate below the transition temperature (ca. 20°C) only dimers of the catalytic subunit are formed, which suggests that only intramolecular crosslinks are formed. In our experiments at 0°C no crosslinking could be demonstrated, although the enzyme activity is inhibited (fig. 9.1b and d). Also in the reaction of the enzyme with Bis at 0°C no crosslinking, intra- or intermolecular, is formed, although the enzyme activity is again inhibited. Reaction of Azo-bis with Na-K ATPase at 0°C does, however, lead to crosslinking. The length of this Azo-bis crosslink will be able to span a 22 Å wide gap between two Na-K ATPase molecules, so intermolecular crosslinking cannot be excluded in this case. Deguchi et al. (1977) have shown that in unfixed Na-K ATPase preparations the enzyme molecules are arranged in clusters, which will increase the likelihood of intermolecular crosslinks.

After crosslinking, with the sulfhydryl reagents, trimers and tetramers of the catalytic subunit are observed. This would seem to point to intermolecular crosslinking, since there are only two catalytic subunits per molecule. However, they may be due wholly or in part to spontaneous polymerization during dissolving in SDS (artifact!), hence their occurrence is no proof for intermolecular crosslinking.

Reaction between N,N' dimethylsuberimidate and Na-K ATPase gives a 180,000 band, and thus appears to result in the expected crosslink between the catalytic and the glycoprotein subunits. Subsequent treatment with Azo-bis or Bis results in the formation of many crosslinked products between the catalytic subunit and the glycoprotein subunit. However, the possibility of intermolecular crosslinks formed by reaction with Azo-bis or Bis make it impossible to determine the number of subunits present in the Na-K ATPase enzyme molecule.

Discrimination between products of intra- and intermolecular crosslinks by SDS gel electrophoresis is also difficult. A reliable distinction between intra- and extramolecular crosslinking reactions might be possible through electronmicroscopic studies of crosslinked preparations. The ratio between free and clustered particles will be affected by intermolecular, but not by intramolecular crosslink reactions.

CHAPTER 10

GENERAL DISCUSSION AND SUMMARY

The enzyme (Na^++K^+) activated ATPase (Na-K ATPase, E.C. 3.6.1.3) plays a role in the active transport of Na^+ and K^+ ions across animal plasma membranes. The enzyme is located in the plasma membrane. It is a complex molecule with a molecular weight of 250,000-280,000, which consists of at least two catalytic subunits, with a molecular weight of 95,000, and either one or two glycoprotein subunits, with a molecular weight of 45,000. The relation between structure and function of this enzyme system has not yet been elucidated (chapter 1). This is at least partly due to the fact that untill recently purification of this enzyme was not possible. We have purified the enzyme from plasma membranes of the rabbit kidney outer medulla, which is an abundant source for this enzyme. The purification method is based on the selective extraction of contaminating membrane proteins by sodium dodecylsulfate (chapter 3). The final preparation is a membrane preparation, which contains more than 90% Na-K ATPase on protein basis.

Various properties of the purified enzyme have been investigated. The overall Na-K ATPase activity and various partial reaction have been studied (methods are described in chapter 2). Some of these partial reaction involve the E_1 conformation of the enzyme (see fig. 1.1), viz. Na^+ dependent phosphorylation by ATP, and Na^+ stimulated ATPase activity. Other partial reactions involve only the E_2 conformation of the enzyme, viz. K^+ stimulated 4-nitrophenylphosphatase activity, and phosphorylation by 4-nitrophenylphosphate or inorganic phosphate. The latter two have been studied in more detail, since they had not yet been investigated in a pure enzyme preparation (chapter 4). All activities, which had previously been demonstrated in impure enzyme preparations, have also been demonstrated in the purified preparation. This means that purification does not induce any essential changes in the enzyme reaction mechanism.

Next an attempt has been made to obtain information about the relation

between structure and function of the enzyme by observing the effects of groupspecific chemical modification on various enzyme parameters. The presence of essential sulfhydryl groups (chapter 5, 6 and 7) and essential arginine groups (chapter 8) is demonstrated. The effects of modification on the various reactions of the Na-K ATPase system have been measured. This could in principle be helpful in interpreting various models for the ATP hydrolysis reaction mechanism. The effects of various cations (Na^+, K^+, Mg^{2+}) and specific ligands (ATP, ADP) on the reactions with the essential groups have been studied. Effects of the cations on the conformation of the enzyme have been established, whereas the ligand studies have given indications for the specific localization of the essential group(s).

The effects of the sulfhydryl reagents N-ethylmaleimide (chapter 5) and 5,5' dithiobis (2-nitrobenzoic acid) (chapter 6) on overall Na-K ATPase activity and partial reactions have been studied under various conditions. By comparing the effects of the two reagents in combination or alone we have been able to classify the various sulfhydryl groups and determine their location on the two types of subunits of the enzyme (chapter 7).

The treatment with either N-ethylmaleimide or 5,5' dithiobis (2-nitrobenzoic acid) shows differences and agreements in inhibition of overall and partial reactions. Agreements exist in the parallel and equal inhibition of the phosphorylation by ATP and the Na-K ATPase activity. Binding of ATP on the high affinity binding site protects against inactivation by both reagents.

Differences in effects are also observed, e.g. in equal and parallel inhibition of Na-K ATPase and K^+-stimulated 4-nitrophenylphosphatase activities by N-ethylmaleimide, whereas the latter activity is inhibited less by 5,5' dithiobis (2-nitrobenzoic acid), and in the effects of ATP or 4-nitrophenylphosphate, under phosphorylating conditions, on inhibition of Na-K ATPase and 4-nitrophenylphosphatase activities by both reagents. There is an essential sulfhydryl group, which can be modified by N-ethylmaleimide, but not by 5,5' dithiobis (2-nitrobenzoic acid).

Titration of the number of sulfhydryl groups under various conditions, has enabled us to distinguish three classes of sulfhydrylgroups on native enzyme:

- Class A, 12 easily accessible sulfhydrylgroups, which react with both N-ethylmaleimide and 5,5' dithiobis (2-nitrobenzoic acid). They are all

located on the catalytic subunit. At least one essential group is included.

- Class B, at least 14 sulfhydryl groups, located in a lipophilic region, which are less accessible and react only with N-ethylmaleimide. They are also located on the catalytic subunit, and include at least one essential sulfhydrylgroup.
- Class C, at most 10 sulfhydrylgroups, reacting neither with N-ethylmaleimide nor with 5,5' dithiobis (2-nitrobenzoic acid). Two of these groups are located on the glycoprotein subunit. It is not known whether any of these groups are essential.

There are 36 sulfhydryl groups per molecule Na-K ATPase, 34 of which are located on catalytic subunits, and 2 on glycoprotein subunit(s).

There are at least 2 different sulfhydryl groups which are essential for Na-K ATPase activity. One belongs to class A, , and one to class B. The class A group is involved in reactions via the E_1 conformation of the enzyme, and may be located in the ATP binding center. There may be another group which is only essential for reaction involving only the E_2 conformation of the enzyme. The one class B essential group reacts with N-ethylmaleimide but not with 5,5' dithiobis (2-nitrobenzoic acid). Alkylation of this group inhibits overall activity and partial reactions equally. This group is not the most reactive sulfhydryl group, at least 3 groups are more reactive.

The presence of an essential arginine group has been demonstrated by means of butanedione, which reacts specifically with arginine residues. The partial reactions, which require the E_1 conformation, are inhibited equally with the Na-K ATPase activity. The partial reactions, which require only the E_2 conformation, are inhibited much less. Binding of ATP, ADP or adenylylimidodiphosphate to their high affinity binding site protects against inactivation. The inhibition rate is lowered by replacement of Na^+ ions by K^+ ions. Inactivation is slowed down by previous reaction with 5,5' dithiobis (2-nitrobenzoic acid). It is concluded that an essential arginine residue is located in the ATP binding centre, possibly quite close to an essential sulfhydryl residue, which is involved in binding of ATP through interaction with its phosphate moiety.

All reactive sulfhydrylgroups and the essential arginine residue are located on the catalytic subunits of the enzyme. Their reactivity depends

on type and concentration of the cations present (Na^+, K^+, Mg^{2+}). This indicates that the subunits undergo conformational changes. At least 4 different conformations can be distinguished. These subunits seem to be very mobile. The biphasic effects of Na^+, K^+ and Mg^{2+} ions on the inhibition of enzyme activity indicate the presence of sites with high affinity ($K_{diss} \leq 1$ mM) and sites with low affinity ($K_{diss} > 5$ mM).

The main result of this study is the evidence for an essential sulfhydrylgroup and an essential arginine residue inside the high affinity ATP binding center, which are both involved in the binding of ATP. Other essential sulfhydrylgroups and arginine residues may be located in the 4-nitrophenylphosphate binding center. The catalytic subunit appears to be rather mobile, and conformational changes of this subunit may be involved in ion transport.

Crosslinking of two catalytic subunits and of a catalytic with a glycoprotein subunit has been achieved through reaction with cupric $(o\text{-phenanthroline})_2$ sulfate, bis maleimidomethyl ether, p-azophenyl N,N' dimaleimide and N,N' dimethylsuberimidate (chapter 9). These findings confirm the presence of two catalytic subunits and at least one glycoprotein subunit per enzyme molecule, but no certainty has been obtained about the presence of one or two glycoprotein subunits.

Het door natrium en kalium ionen gestimuleerde ATPase (Na-K ATPase, E.C. 3 6.1.3) speelt een rol in het actieve tranport van natrium en kalium ionen over het celmembraan. Het enzym is in dit membraan gelegen en heeft een moleculair gewicht van ongeveer 250.000. Het bestaat uit meerdere subunits. Daarvan zijn er twee zg. catalytische subunits met een moleculair gewicht van 95.000 en één of twee glycoproteine subunits met een moleculair gewicht van 45.000. In hoofdstuk 1 worden de fysiologische betekenis van dit enzym, het voorkomen en het reactiemechanisme besproken.

Het verband tussen de structuur en de functie van dit enzymsysteem is nog niet opgehelderd. Gedeeltelijk komt dit doordat tot voor kort het enzym niet gezuiverd kon worden. Wij hebben het enzym gezuiverd uit plasma membranen van de buitenste laag van het merg van de konijnenier. Deze zuivering is gebaseerd op de extractie van andere membraan eiwitten met behulp van een detergens (natrium dodecylsulfaat) Deze methode levert membraan fragmenten, waarin het enzym Na-K ATPase meer dan 90% van het totale eiwitgehalte omvat. Deze zuivering en andere (niet geslaagde) pogingen tot zuivering van het Na-K ATPase zijn beschreven in hoofdstuk 3.

Na de zuivering hebben we eerst verschillende enzym parameters bepaald volgens de in hoofdstuk 2 beschreven methoden. Zowel de Na-K ATPase activiteit als geheel en de verschillende deelreacties zijn hierbij bestudeerd. Deze deelreacties kunnen onderscheiden worden in reacties, die betrekking hebben op de eerste fase resp. de tweede fase van de ATPase reactie. Deelreacties van de eerste fase zijn de fosforylering uit ATP en de Na^+ gestimuleerde ATPase activiteit. Bij deze reacties verkeert het enzym in een bepaalde conformatie, de zg. E_1 vorm (zie fig. 1.1). Deelreacties van de tweede fase zijn de K^+ gestimuleerde 4-nitrofenylfosfatase activiteit, en de fosforyleringen uit 4-nitrofenylfosfaat of anorganisch fosfaat. Bij deze reacties verkeert het enzym in een andere conformatie, de zg. E_2 vorm. De K^+ gestimuleerde 4-nitrofenylfosfatase activiteit en de fosforylering door 4-nitrofenylfosfaat zijn diepgaander bestudeerd, aangezien dit voor een gezuiverd preparaat nog niet gedaan was Alle deelreacties die in onzuivere preparaten aangetoond waren, zijn

in het gezuiverde preparaat teruggevonden. Dit betekent dat het reactiemechanisme van het enzym in essentie ongewijzigd is gebleven door de zuivering. De resultaten van deze experimenten zijn gepresenteerd in hoofdstuk 4.

Een van de mogelijkheden om meer te weten te komen over het verband tussen de structuur en de functie van het enzym, is het bestuderen van de effecten van een chemische modificatie van aminozuur residuen op de verschillende enzym parameters. Op deze wijze zijn met specifieke reagentia zowel sulfhydryl groepen (zie hoofdstuk 5 en 6) als arginine residuen (zie hoofdstuk 8) aangetoond, die essentieel zijn voor de Na-K ATPase activiteit. De gevolgen van modificatie van deze groepen voor de activiteit als geheel zowel als voor de deelreacties zijn verschillend, afhankelijk van het gebruikte reagens. Dit zou ons in principe kunnen helpen bij het ophelderen van het reactie mechanisme van de hydrolyse van ATP.

Modificatie van sulfhydryl groepen met N-ethylmaleimide remt de ATPase reactie als geheel en de verschillende deelreacties in gelijke mate, terwijl na reactie met 5,5' dithiobis (2-nitrobenzoezuur) de K^+ gestimuleerde 4-nitrofenylfosfatase activiteit minder geremd is dan de Na-K ATPase activiteit. ATP kan de remming door zowel N-ethylmaleimide als 5,5' dithiobis (2-nitrobenzoezuur) tegengaan. Verschillen in afscherming zijn echter gevonden, welke afhankelijk zijn van de condities waaronder de afscherming bepaald is. De remming blijkt vergroot of verkleind te kunnen worden door de aanwezigheid van natrium, kalium en magnesium ionen, maar de invloeden zijn verschillend, afhankelijk van de gebruikte remmer en de ionconcentratie. Deze resultaten zijn beschreven in hoofdstuk 5 voor N-ethylmaleimide, en in hoofdstuk 6 voor 5,5' dithiobis (2-nitrobenzoezuur).

In totaal zijn er 36 sulfhydrylgroepen aanwezig in het enzym. Daarvan zijn er 34 aanwezig in de katalytische subunit en 2 in de glycoproteine subunit. Het totale aantal sulfhydryl groepen wordt slechts gevonden nadat het enzym is opgelost in een detergens. Alle sulfhydryl groepen in het natieve enzym, die reageren met N-ethylmaleimide en 5,5' dithiobis (2-nitrobenzoezuur), liggen in de katalytische subunit. We hebben de sulfhydrylgroepen kunnen onderbrengen in drie klassen:

- Klasse A, 12 gemakkelijk toegankelijke groepen. Deze reageren zowel met 5,5' dithiobis (2-nitrobenzoezuur) als met N-ethylmaleimide. Deze groepen liggen allen op de katalytische subunit, en omvatten minstens één essentiele groep.

- Klasse B, minstens 14 minder toegankelijke groepen, vermoedelijk gelegen in een hydrofobe omgeving Deze groepen reageren alleen met N-ethylmaleimide. Zij liggen allen in de katalytische subunit, en omvatten tenminste één essentiele groep
- Klasse C, maximaal 10 zeer moeilijk toegankelijke of ontoegankelijke groepen. Deze groepen reageren niet met N-ethylmaleimide en 5,5' dithiobis (2-nitrobenzoezuur), tenzij het enzym is opgelost in een detergens. Hiervan zijn er 2 op de glycoproteine subunit gelegen.

Tenminste twee verschillende sulfhydryl groepen, die essentieel zijn voor de Na-K ATPase activiteit, zijn gevonden Eén van deze groepen behoort tot klasse A. Deze groep speelt een rol bij reacties via de E_1 conformatie van het enzym, en lijkt gelegen te zijn in de bindingsplaats voor ATP. Er is mogelijk nog een tweede essentiele groep in klasse A, welke alleen activiteitsbepalend is voor reacties die via de E_2 vorm van het enzym verlopen, zoals de K^+ gestimuleerde 4-nitrofenylfosfatase activiteit. De essentiele groep uit klasse B reageert alleen met N-ethylmaleimide. Modificatie van deze groep remt de enzym activiteit als geheel en de deelreacties in gelijke mate. Deze essentiele groep is niet de meest reactieve groep, er zijn tenminste 3 sulfhydryl groepen die sneller reageren met N-ethylmaleimide De experimenten, die geleid hebben tot de klassificatie van de aanwezige sulfhydrylgroepen, zijn beschreven in hoofdstuk 7.

Er is ook een arginine residu aangetoond, dat bepalend is voor de enzymactiviteit. Reactie van deze groep met 2,3 butaandion leidt tot remming van de enzymactiviteit. De enzymactiviteit als geheel, en de fosforylering door ATP worden sterker geremd dan de K^+ gestimuleerde 4-nitrofenylfosfatase activiteit en de fosforylering door anorganisch fosfaat. Zoals bij de remming door modificatie van sulfhydrylgroepen, kan ook in dit geval de remming worden tegengegaan door ATP. De remming door butaandion wordt beínvloed door de aanwezigheid van natrium of kalium ionen. De effecten van butaandion op de Na-K ATPase activiteit en de deelreacties staan beschreven in hoofdstuk 8

Uit deze modificatie experimenten kunnen de volgende conclusies getrokken worden voor het reactiemechanisme van het enzym en het verband tussen structuur en functie

1. Een sulfhydrylgroep en een arginine residu zijn aanwezig in de ATP bindingsplaats met hoge affiniteit, en zij zijn betrokken bij de binding van ATP

2. De katalytische subunits zijn zeer flexibel, en kunnen tenminste 4 verschillende conformaties aannemen.
3. Natrium, kalium en magnesium ionen hebben elk tenminste twee bindingsplaatsen.
4. Er zijn nog andere activiteitsbepalende sulfhydryl groepen en arginine residuen naast die welke in de ATP bindingsplaats gelegen zijn.

Pogingen zijn ondernomen om het aantal subunits van het Na-K ATPase complex vast te stellen. Deze experimenten zijn vermeld in hoofdstuk 9. Met behulp van de reagentia koper (o-fenanthroline)$_2$ sulfaat, bis maleimidomethyl ether en p-azofenyl N,N' dimaleimide is het gelukt de katalytische subunits aan elkaar te binden via de sulfhydrylgroepen. Het blijkt dat door deze reactie de Na-K ATPase activiteit geremd wordt Zowel de aaneenhechting als de remming van de Na-K ATPase activiteit worden beïnvloed door de reactietemperatuur en door de aanwezigheid van ATP.

Door de reactie met N,N' dimethylsuberimidate zijn een katalytische en een glycoproteine subunit aan elkaar gehecht via de vrije aminogroepen. Opeenvolgende reacties met dimethylsuberimidaat en p-azofenyl bismaleimide of bismaleimidomethyl ether leveren verscheidene polymeren op. Aangezien het onmogelijk is vast te stellen in hoeverre deze aaneenhechtingen alleen binnen één enzymcomplex of ook tussen verschillende enzymcomplexen plaatsvinden, zijn we thans nog niet in staat het aantal subunits binnen het enzymcomplex vast te stellen, met name het aantal glycoproteine subunits.

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Lipid requirement of the membrane Na-K ATPase system.
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Yoshida, H., Nagai, K., Ohashi, T. and Nakagawa, Y. (1969) Biochim. Biophys. Acta 171, 178-185.
K^+ dependent phosphatase activity, observed in the presence of both ATP and Na^+.

CURRICULUM VITAE

De schrijver van dit proefschrift is geboren op 26 april 1948 te Eindhoven. Na het behalen van het eindexamen Gymnasium β (1966, Gymnasium Augustinianum, Eindhoven) werd begonnen met de studie scheikunde aan de Katholieke Universiteit te Nijmegen. In januari 1970 werd het kandidaatsexamen (S2) en in november 1973 het doctoraal examen Scheikunde afgelegd (Hoofdrichting: Biochemie; Bijvak: Kinetiek en Katalyse; Capita: Instrumentele Methoden I en II, Quantumchemie en Ethiek).

Vanaf september 1973 tot en met december 1977 was hij aangesteld als wetenschappelijk medewerker aan de afdeling Biochemie van de Faculteit der Geneeskunde en Tandheelkunde (Katholieke Universiteit te Nijmegen), waar naast de bijdrage aan het onderwijs aan pre-kandidaats studenten in de geneeskunde en de tandheelkunde en doctoraalstudenten scheikunde en biologie, het onderzoek voor dit proefschrift verricht werd.

In 1977 is hij getrouwd met Elsa Messelink.

STELLINGEN

I

Een verhoging van de cyclisch GMP concentratie in de acineuze cel van de pancreas ten gevolge van een stimulatie van de enzymsecretie bewijst niet dat cyclisch GMP een rol speelt bij de stimulus secretie koppeling.

A. Haymovits en G.E. Scheele (1976)
Proc. Natl. Acad. Sci. 73, 156-160.
J. Diamond en R.A. Janis (1978)
Nature 271, 472-473.

II

Isenman en Rothman verwaarlozen ten onrechte de mogelijkheid van een resorptie langs paracellulaire weg als verklaring van het schijnbare transport van α-amylase door het baso-laterale membraan van de acineuze cel in de alvleesklier.

L.D. Isenman en S.S. Rothman (1977)
Proc. Natl. Acad. Sci. 74, 4068-4072.

III

De conclusie dat het (H^++K^+) geactiveerde ATPase in vesikels van maag mucosa membranen een dimeer enzym is is niet gerechtvaardigd.

H. Chang, G. Saccomani, E. Rabon, R. Schackmann en G. Sachs (1977)
Biochim. Biophys. Acta 464, 313-327.

IV

Een knik in een Arrhenius grafiek (log activiteit vs. reciproke waarde van de reactietemperatuur in graden Kelvin) voor de reactie van een membraan gebonden enzym hoeft niet te duiden op een verandering van de Arrhenius activeringsenergie.

L. Thilo, H. Träuble en P. Overath (1977)
Biochemistry 16, 1283-1290.

V

De toekenning van een struktuur door Khedija et al. aan de door hen geisoleerde trimethine kleurstoffen is onzorgvuldig en vermoedelijk onjuist.

H. Khedija, H. Strzelecka en M. Simalty (1973)
Bull. Soc. Chim. France. 1, 218-223

VI

De conclusie van Hazum et al., dat de geringe biologische activiteit van de door hen gesynthetiseerde analoga van het luteinizing-hormone releasing hormone voortkomt uit de verminderde neiging tot de vorming van charge-transfer complexen via de indool ring van tryptofaan-3, is onvoldoende gefundeerd.

E. Hazum, M. Fridkin, R. Meidan en Y. Koch (1977)
Eur. J. Biochem. 79, 267-273

VII

De door Bachmann en Challoner gegeven waarden voor de glucose opname in preparaten van rattelever membranen zijn te laag.

W. Bachmann en D. Challoner (1976)
Biochim. Biophys. Acta 443, 254-266.

VIII

Bij het bestuderen van de intermediaire stofwisseling dient rekening gehouden te worden met een samenwerking van verschillende organen.

IX

De suggestie, dat de fosforylering van het Na-K ATPase meer sulfhydryl groepen voor reactie toegankelijk maakt, is onvoldoende gefundeerd.

W.M. Hart en E.O. Titus (1973)
J. Biol. Chem. 248, 4674-4681
C.A. Takeguchi, U.E. Honegger, W.W. Holland en E.O. Titus (1976)
Life Sci. 19, 797-806
Dit proefschrift, hoofdstukken 5, 6 en 7.

X

Het reactiemechanisme, zoals dit is voorgesteld door Sweadner, ter verklaring van een intramoleculaire verknoping door het monofunctionele reagens ethylaceetimidaat, is niet aannemelijk.

K.J. Sweadner (1977)
Biochem. Biophys. Res. Commun. 78, 962-969.
D.T. Browne en S.B.H. Kent (1975)
Biochem. Biophys. Res. Commun. 76, 126-138

XI

Het onderscheid tussen hormonen en neurotransmitters vervaagd steeds meer.

K. Uvnäs-Wallensten, J.F. Rehfeld, L.-I. Larsson en B. Uvnäs.(1977)
Proc. Natl. Acad. Sci. USA 74, 5707-5710.

XII

De vorming van dimeren van de katalytische peptide van het Na-K ATPase door reactie in aanwezigheid van koper fenantroline sulfaat bij temperaturen beneden de z.g. overgangstemperatuur (20° C) is geen bewijs voor een intramoleculaire reactie.

S.M. Liang en C.G. Winter (1977)
J. Biol. Chem. 252, 8278-8284
Dit proefschrift, hoofdstuk 9.

XIII

Bloch verwaarloost ten onrechte de mogelijkheid van een reactie van N-ethylmaleimide met meer lipofiele sulfhydryl groepen.

R. Bloch (1974)
J. Biol. Chem. 249, 1814-1822
Dit proefschrift, hoofdstuk 7.

XIV

De redactie van de promotieregelingen van deze universiteit maakt het onmogelijk naast de promotor een co-referent in het proefschrift te vermelden op de in deze regelingen voorgeschreven wijze.

Promotieregelingen van de Katholieke Universiteit Nijmegen (okt. 1976) , Hoofdstuk 5, artikels 22.1 en 22.3.

B.M. Schoot, Nijmegen, 23 februari 1978.